

CHROMATIN STRUCTURE
OF THE rRNA GENES
OF *DICTYOSTELIUM DISCOIDEUM*:
STUDIES ON THE
5' STRUCTURAL BOUNDARY

INAUGURAL-DISSERTATION

zur Erlangung der philosophischen Doktorwürde
vorgelegt der
Philosophischen Fakultät II
der Universität Zürich

von
BERNHARD BETTLER
aus Zweisimmen (BE)

Begutachtet von den Herren
Prof. Dr. R.W. Parish
Prof. Dr. Th. Koller

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1. INTRODUCTION

Considerable experimental evidence suggests that two fundamentally different classes of mechanisms are involved in eukaryotic gene expression: one which renders a gene available for transcription (the process of gene activation and gene commitment) and a second which induces and regulates the actual expression of the potentially active gene. Establishment of a general nuclease- (DNase I -) sensitivity appears to be one of the important developmental steps in the commitment of regions of chromatin to transcription, since the nuclease-sensitivity has been correlated with the differentiated state of the cell rather than transcriptional activity per se (e.g. Stalder et al., 1980a; reviews by Weisbrod, 1982; Cartwright et al., 1982; Conkline and Groudine, 1984; Reeves, 1984). The preferential nuclease-sensitivity presumably reflects a removal or an alteration of nucleosomes (e.g. Karpov et al., 1984; Labhart and Koller, 1982; Levy and Noll, 1981; Cohen and Sheffrey, 1985; Davis et al., 1983) and/or a change in the higher order structure of chromatin (Stalder et al., 1980b; Weintraub, 1984; Smith et al., 1984).

Villeponteau et al. (1984) proposed that the altered structure around active genes is induced and maintained by the continuous DNA supercoiling action of a topoisomerase II. In the absence of this superhelical tension, active chromatin reverts to a less DNase I-sensitive ground state.

The nuclease-sensitivity of most genes analysed is not restricted to the coding region, but extends far upstream and downstream (e.g. Stalder et al., 1980b; Weintraub et al., 1981; Lawson et al., 1980, 1982; Bellard et al., 1980, 1982). It has been postulated that the chromatin fiber is packaged either into nuclease-sensitive or -insensitive domains corresponding to the loops both of metaphase chromosomes and interphase nuclei. Expressible genes would then be included in nuclease-sensitive, repressed genes in nuclease-resistant loops (Lawson et al., 1982). If organization of transcriptionally poised genes into domains is a common phenomenon, specific sequences might be expected to play a structural and functional role. Repetitive sequences (Lawson et al., 1982) and A/T rich linkers (Moreau et al., 1982) could, for example, define the limits of the active (nuclease-sensitive) domain. Specific DNA attachment sites on the nuclear scaffold have been identified in nontranscribed spacer regions at the base of chromatin loops (Mirkowitch et al., 1984; Cockerill and Garrard, 1986). It was proposed that these sites are members of the A/T rich sequence elements flanking active chromatin domains. It was further demonstrated that the matrix association region contains topoisomerase II sites (Udvardy et al., 1985; Cockerill et al., 1986; Gasser and Laemmli, 1986) and that this enzyme is located at the bases of the DNA loops of mitotic chromosomes (Earnshaw and Heck, 1985). Cockerill and Garrard (1986) proposed a model in which the matrix association regions of chromosomal loops may provide both a means of

anchoring linear molecules and a topoisomerase swivel for the introduction and maintenance of torsional stress.

The localization of the boundaries of nuclease-sensitive chromatin domains at the sequence level seems to be a prerequisite for an understanding of the mechanism underlying chromatin organization. It is likely that the boundaries of functional domains are target sites for the mechanism that initiates, maintains and possibly modulates the altered state. If domains defined by nuclease-sensitivity indeed correspond to radial loop domains, one might expect to find matrix-interaction sequences and topoisomerase II sites at the limits of active chromatin (see above).

A general sensitivity to DNase I (e.g. Weintraub and Groudine, 1976) or micrococcal nuclease (e.g. Palen and Cech, 1983) is a feature common to transcriptionally active mRNA and rRNA genes (Weintraub and Groudine, 1976; for rRNA genes see e.g. Ness et al., 1983; Palen and Cech, 1983; 1984; Davis et al., 1983; Prior et al., 1983, Udvardy et al., 1984). Extensive nucleosome-free regions are seen on active *Xenopus* ribosomal genes and their spacers (Labhart and Koller, 1982). Hence, mRNA and rRNA genes share the fundamental problem of establishing and maintaining an "open" chromatin state. The detailed knowledge accumulated about the structural organization of the rRNA genes of *D. discoideum* make them an ideal system for the localization of domain boundaries and the analysis of structural transitions accompanying cell differentiation. Nucleoli constitute a morphologically and functionally distinct region of interphase nuclei, containing 90 copies of a 88 kb extrachromosomal, linear palindromic rDNA molecule. Electron microscopy and biochemical data from isolated vegetative cell nucleoli suggest a model in which the nuclease-sensitive transcribed rDNA is thought to be devoid of nucleosomes. Psoralen-crosslinking in nucleoli, followed by electron microscopy of purified DNA molecules, indicated that the site where the chromatin structure changes from its active to its inactive conformation is 2 to 3 kb upstream and downstream from the transcribed sequences. No electron microscopic data is available for differentiating slug cells, where the synthesis of rRNA is reduced by 80% and cell division has stopped completely (Soll and Sussman, 1973; Ness et al., 1986). However, it has been shown that the rRNA coding region reverts to a nucleosomal arrangement (Ness et al., 1983) though a preferential nuclease-sensitivity as compared with bulk chromatin is retained (Ness et al., 1986; this work, unpublished observations).

In the studies reported here we have analysed both by psoralen-crosslinking and nuclease digestions the chromatin structure of the 5' non-transcribed spacer and the coding regions of the rRNA genes in *D. discoideum*. We define a sharp structural boundary between nuclease-sensitive and -resistant chromatin domains 2.3 kb upstream from the rRNA initiation site. Site-specific topoisomerase-like activity maps to two of the three strong micrococcal nuclease/DNase I

hypersensitive sites upstream from the coding region. Putative topoisomerase sites of action occur at the nuclease hypersensitive site about 200 bp upstream from the start site of transcription. This rDNA region contains inverted and direct repeats of a high affinity topoisomerase I binding sequence clustered at DNase I hypersensitive sites in *Tetrahymena* r-chromatin (Bonven et al., 1985). Topoisomerase-like activity in the rDNA of *D. discoideum* associated with a second hypersensitive site maps to the 5' structural boundary. We thus propose that topoisomerase-like activity denotes the upstream limit of the nuclease-sensitive domain. This and other findings are included in a structural model for the 5' end of the rRNA genes incorporating changes related to transcriptional activity.

2. MATERIAL AND METHODS

2.1 RESTRICTION ENDONUCLEASE MAPPING OF rDNA

Cleavage sites of restriction endonucleases on the rDNA around the Eco RI site between Eco RI fragments II and V were mapped by Southern blotting. Hybridization probes were gel-purified nucleolar Eco RI fragment II and the ~1.6 kb Eco RI-Ava II insert of pEcoV.

2.2 PLASMIDS

Plasmids pEcoV, pEcoVII and pMG6 are gifts from P. J. Ness (Ness et al., 1983, 1986). Plasmids used for cloning were pBR322/328 (Soberon et al., 1980; Sutcliffe, 1978/79), and pUC12/13/18/19 (Vieira and Messing, 1982; Messing, 1983). pPvE is derived from an Eco RI-Pvu II double digest of nucleolar DNA and maps to the rRNA gene proximal end of Eco RI fragment II. pPvH and pSE are subclones of pPvE. pEP, pEPv, pMG6 and pPP are subclones of pEcoV. pHPv is derived from pEPv; pAA and pAS from pEP; pPS and pSB from pPP; pSaB and pTE from pMG6, respectively. A characterization of the plasmid inserts and the cloning sites is given in section 3.1.2. Ava II restriction cleavage sites were made blunt ended by filling in with the Klenow fragment of polymerase I and cloned into the Sma I site of the pUC polylinker. All plasmids were grown in *E. coli* HB101. Restriction fragments used for ligation and nick translation were recovered from low gelling temperature agarose (SIGMA, type VII) gels. The cloning procedures applied were according to Maniatis et al. (1982). Location of all plasmid inserts used in the experiments is summarized in Fig. 1B.

2.3 ISOLATION OF NUCLEI, NUCLEOLI AND DNA

The growing of *D. discoideum* cells, axenic strain AX-2, the purification of nuclei and nucleoli and the extraction of DNA were performed as described (Widmer et al., 1979; Ness et al. 1983), except that lysis with Triton-X-100 was carried out at 0°C and the nuclear/nucleolar pellet was washed in 20 mM Tris-HCl (pH 7.6), 0.5 mM CaCl₂.

Nuclei to be used in the topoisomerase experiments were obtained by washing cells with NB buffer (10 mM Tris-HCl, pH 7.2, 3 mM CaCl₂, 1mM MgCl₂, 100 mM sucrose). Cells were taken up in 200 ml NB buffer and lysed by adding 0.3% Nonidet P-40 and stirring on ice. Nuclei were obtained by centrifuging at 2000 g for 5 min at 4°C (Gocke et al., 1983).

The induction of developmental stages was as described by Ness et al. (1983), but cells were plated onto agarose (FLUKA) and not agar. Agar inhibits restriction endonuclease digestion of

purified DNA unless additional purification steps are carried out.

DNA was quantified by spotting aliquots onto agarose plates containing 1µg/ml ethidium bromide.

2.4 TREATMENT OF NUCLEI WITH NUCLEASES

Washed nuclei were resuspended in the digestion buffer (0.25 M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM Tris-HCl (pH 7.4), 0.5 mM dithiothreitol, 3 mM MgCl₂, 0.5 mM CaCl₂) at a concentration of 10⁹ nuclei/ml. Prior to treatment with nuclease all samples were prewarmed for 1 min at the incubation temperature of 25⁰ C. DNase I was added to the nuclear suspension to a final concentration of 80 U/ml for vegetative and 50 U/ml for slug nuclei. Incubation with micrococcal nuclease (MNase) was with 12.5 U/ml for vegetative and 5 U/ml for slug nuclei, respectively. Samples were taken at various time points. The digestion times in the experiments described were 45 s and 2 min for both DNase I and MNase. Endogenous nuclease activity was determined by incubation of the nuclei at 25⁰ C without added enzyme for 2 to 20 min. All reactions were stopped by addition of EDTA to 20 mM and sodium dodecyl sulphate (SDS) to 0.4%. Purification of DNA was as described (Ness et al., 1983). For indirect end-labelling experiments the nuclease digested DNA was secondarily cleaved to completion with restriction enzyme prior to gel electrophoresis. DNA was digested with restriction endonucleases according to manufacturers recommendations.

2.5 TREATMENT OF DEPROTEINIZED DNA WITH NUCLEASES

"Instant" DNA was extracted from whole cells to minimize endogenous nuclease activity in the naked DNA samples. Exponential growing cells were harvested at 4⁰ C and resuspended in ice cold TE (10 mM Tris-HCl (pH 7.6), 1 mM EDTA). After addition of 10 volumes of 4% sarcosyl, 0.1 M EDTA (pH 8.0), 20 mM Tris-HCl (pH 8.0) at 65⁰ C and incubation for 5 min at this temperature, the DNA was purified on cesium chloride/ethidium bromide gradients. For nuclease treatment, DNA was resuspended (1mg/ml) as described and incubated for 30 s to 2 min at 0⁰ C. DNA was incubated with 10 U/ml DNase I and 7.5 U/ml micrococcal nuclease. Following nuclease digestion, the reaction was stopped and the DNA repurified and restricted as described in the previous section.

2.6 PSORALEN-CROSSLINKING

The psoralen treatment was essentially as described by Sogo et al. (1984). Nuclei were washed and resuspended in 20 mM Tris-HCl (pH 7.6), placed in open petri dishes on ice and 0.01

vol. 4,5',8-trimethylpsoralen (SIGMA) stock solution in ethanol (1 mg/ml) was added. Samples were irradiated for 4 h with long wavelength ultraviolet light. Free psoralen is destroyed by irradiation with UV-light, so that portions of the psoralen stock suspension were added every hour (0.04 end vol.). An excess of restriction endonucleases was used to cut psoralen-crosslinked DNA. Naked DNA was cleaved prior to the application of psoralen, since it was impossible to cut the DNA to completion after heavy crosslinking (Carlson et al., 1982).

2.7 SDS-INDUCED CLEAVAGES (TOPOISOMERASE ASSAY)

Treatments are according to Gocke et al. (1983). For the SDS-induced cleavages, nuclei were incubated at 25⁰ C; SDS and EDTA were added together to give 1% (w/v) and 10 mM respectively. After gently mixing, NaCl was added to give 0.8 M. For the "NaCl" treatment, nuclei were cooled on ice and ice cold NaCl and EDTA (pH 7.2) were added together to give end concentrations of 0.8 M and 10 mM, respectively. After 5 min at 0⁰ C, SDS was added to give 1% end concentration. Following proteinase K digestion (200 µg/ml), the DNA was purified with standard methods. The samples shown in Fig. 14 were kindly provided by P. J. Ness.

2.8 GEL ELECTROPHORESIS AND BLOTTING PROCEDURE

DNA from chromatin and deproteinized DNA digests were always run on the same gel. Molecular weight markers were Eco RI-Hind III standards of λ DNA, the 123 bp BRL ladder and Hpa II digested pBR322. Gels with psoralen samples were irradiated at 254nm before blotting in order to reverse the crosslinking (Sogo et al., 1984). Agarose gels (0.75 - 2.3%, w/v), 25cmx20cmx0.3cm, were run in TBE electrophoresis buffer (0.089 M Tris-borate, 0.089 M boric acid and 0.008 M EDTA) for 18 h at 4⁰ C, transferred onto DBM-paper by the method of Southern (1975) as described by Wahl et al. (1979). Polyacrylamide gels (4 - 6%, w/v) were prepared according to Maniatis et al. (1982). DBM-paper was prepared according to Alwine et al. (1979) using N-(3-nitrobenzyl-oxyethyl)-pyridinium chloride (BDH). The DBM-paper was activated immediately prior to the overnight transfer at 4⁰ C.

2.9 HYBRIDIZATION

Plasmid DNA and gel-purified restriction fragments (~50 ng) were labelled by nick translation with (α-³²P) dCTP (3000Ci/mM, Amersham) using an Amersham nick translation kit. Specific activities of up to 10⁹ cpm/µg were obtained. Prehybridization and hybridization were carried out at 68⁰ C in 6xSSC (SSC is 0.15 M - NaCl, 0.015 M - sodium citrate), 1xDenhardt's (0.02%, w/v,

each of Ficoll, polyvinylpyrrolidone and bovine serum albumin), 1% glycine, 0.25 mg sonicated calf thymus DNA / ml and 0.1% SDS. Incubation with the hybridization solution was overnight, although small DNA fragments were hybridized up to 20 h. The paper was washed in 0.2xSSC, 0.2% SDS at 68⁰ C. Blots were exposed to Fuji X-Ray film for 12 - 23h with or without a Tungsten intensifier screen. To reuse the DBM- paper it was stripped three times for 1 min in boiling water and prehybridized as described above.

3. RESULTS

3.1 RESTRICTION MAP AND CLONED FRAGMENTS OF RIBOSOMAL DNA (rDNA)

A map of rDNA is shown in Fig. 1. The rRNA genes are located on ~90 kb of linear, extrachromosomal, palindromic molecules; there are about 90 rDNA dimers per haploid genome. Each rDNA half-repeat contains two transcription units. One transcription unit encodes the 36S rRNA precursor, which is processed to yield the mature 26S, 17S and 5.8S rRNAs. The 36S rRNA genes have an external (ETS) and two internal (ITS) transcribed spacer regions. The 5S rRNA is encoded by a separate transcription unit located 3' to the 36S rRNA genes, towards the telomer. The precise position and the direction of transcription of the 5S rRNA genes are unknown (Cockburn et al., 1976, 1978; Maizels, 1976; Frankel et al., 1977, Loomis, 1982; Hoshikawa et al. 1983; Ozaky et al., 1984). In our experiments we were only interested in the 5' non-transcribed spacer and the coding region of the 36S rRNA genes. We shall therefore be referring to the 36 rRNA genes as rRNA genes. The rRNA precursor initiation site was mapped by Hoshikawa et al. (1983). pEcoV, pEcoVII and pMG6 were already available rDNA clones derived from *D. discoideum* strain AX-2, which were kindly provided by P. Ness (Ness et al., 1983; 1986) (Fig. 1B).

3.1.1 RESTRICTION ENDONUCLEASE MAPPING OF rDNA

To analyse the chromatin structure by the indirect end-labelling method (Nedospasov and Georgiev, 1980; Wu, 1980) we needed to clone small rDNA fragments from various parts of the rDNA molecule. At first, we wanted to establish a more detailed restriction map upstream from the rRNA gene, around the Eco RI site between Eco RI fragments II and V. With this aim, we made a Southern blot analysis of single and double restricted nucleolar rDNA, the second restriction enzyme in the double digests always being Eco RI. We size-fractionated the restricted DNA for the Southern blot analysis both on 0.75% and 2.3% agarose gels to determine new restriction sites over a wide range of rDNA sequences. Fig. 2 shows a hybridization with the gel-purified, terminal 1.6 kb Eco RI-Ava II fragment of pEcoV, which is located upstream from the rRNA transcriptional start site. Fig. 3 shows the same blots as presented in Fig. 2, now hybridized with the gel-purified Eco RI fragment II which was derived from purified nucleolar DNA.

All the newly determined restriction endonuclease sites which were used in the experiments described below are included in the restriction map of Fig. 1A.

3.1.2 CLONING OF rDNA FRAGMENTS

On the basis of our mapping, we cloned the plasmids pPvE (3.0 kb Pvu II-Eco RI insert cloned into pBR328 Pvu II-Eco RI) and pEPv (1.2 kb Eco RI-Pvu II cloned into pBR328 Eco RI-Pvu II) (Fig. 1 B). pPvH (~200 bp Pvu II-Hpa II insert cloned into pUC13 Sma I-Acc I) and pSE (2.2 kb Sac I-Eco RI insert cloned into pUC12 Eco RI-Sac I) were derived from pPvE. pHPv (~200 bp Hpa II-Pvu II insert cloned into pBR328 Acc I-Pvu II and pUC18 Eco RI-Sma I) was derived from pEPv. Fig. 3 shows a size estimation of the inserts of pPvH and pHPv. Nuclear DNA was double digested with Pvu II and Hpa II, fractioned on a 4% polyacrylamide gel, blotted onto DBM paper and hybridized with pPvH and pHPv, respectively. The pPvH insert contains about 240 bp, the pPvH insert about 230 bp. A preliminary analysis of pPvE implies that a second Sac I site is located either just to the right or just to the left of the Hpa II site in the pPvE insert. This Sac I site is not included in the map of Fig. 1A.

The rDNA has recently been sequenced by Ozaky et al. (1984) from the Ava II site 703 bp upstream to the Eco RI site 6970 bp downstream from the rRNA initiation site, and a complete restriction map of this rDNA for *D. discoideum* strain A3 is available. Since our limited restriction site mapping of rDNA in *D. discoideum* strain AX-2 revealed some deviations from the published sequence and Eco RI fragments II, III and V exhibit DNA polymorphism (Welker et al. 1985), we made a Southern blot analysis prior to the cloning of small rDNA fragments in sequenced rDNA regions. Nuclear AX-2 DNA was restricted with various enzymes, size-fractioned on 4% polyacrylamide gels and blotted onto DBM paper. Fig. 4 shows a hybridization with cloned pPP (817 bp Pst-Pst insert cloned into pBR322 Pst I, the insert is derived from pEcoV). The resulting hybridization pattern was in total agreement with the published sequence of *D. discoideum* strain A3 (Hoshikawa et al. 1983) (Fig. 5). This also proved to be the case for the Southern blot analysis of pMG6 (Fig. 6). Some weakly hybridizing bands are due to incomplete digestion of the genomic DNA. We subcloned pPS (260 bp Pst I-Sac I insert cloned into pUC18 Pst I - Sac I) and pSB (222 bp Sac I-Bgl II insert cloned into pUC12 Sac I-Acc I) from pPP. We further subcloned pSaB (212 bp Sal I-Bgl II insert cloned into pUC12 Sal I-Acc I) and pTE (206 bp Taq I-Eco RI insert into pUC12 Eco RI-Acc I) from pMG6. To obtain hybridization probes for the non-transcribed spacer region immediately upstream from the rRNA precursor initiation site, we have cloned pEP (~2600 bp Eco RI-Pst I insert cloned into pBR322 Eco RI-Pst I) and from this plasmid we have subcloned pAA (703 bp Ava II-Ava II insert, made blunt ended using the Klenow-fragment of polymerase I, cloned into pUC18 Sma I) and pAS (135 bp Ava II-Sac I insert, Ava II site made blunt ended using the Klenow-fragment of polymerase I, cloned into pUC18 Sma I-Sac I).

3.2 CHROMATIN STRUCTURE OF THE rDNA

The rRNA genes are developmentally regulated; in vegetative cells the rRNA synthesis rate is high and, subsequently, it is reduced by 70 - 80% in 15 h differentiated slug cells (Soll and Sussman, 1973; Ness et al., 1986). We wished to examine changes in the chromatin structure in different transcribed and non-transcribed regions at the 5' end of the rRNA genes during development. Two different approaches were chosen, namely psoralen-crosslinking (for a review see Cimino et al., 1985) and nuclease digestion of isolated nuclei.

3.2.1 CHROMATIN STRUCTURE ANALYSED BY PSORALEN-CROSSLINKING

Trimethylpsoralen is a DNA intercalating agent which monitors the accessibility of the DNA in chromatin. The extent of psoralen-crosslinking in chromatin DNA is diagnostic for the structure of chromatin. In *D. discoideum*, psoralen-crosslinking has been used to study the chromatin structure of the rRNA genes in vegetative cells by electron microscopy and further to examine changes in chromatin organization during development by an electrophoretic band shift assay (Sogo et al., 1984). Psoralen-crosslinking in nucleoli of vegetative cells followed by electron microscopy of purified DNA molecules allowed mapping of the sites where the chromatin structure changes from its active to its inactive conformation. These sites were found to be 2 to 3 kb upstream and downstream from the transcribed sequences (Sogo et al., 1984). We have used the electrophoretic band shift approach (Carlson et al., 1982) to map more accurately the 5' structural boundary of the active rDNA chromatin domain. We further wished to examine how chromatin regions adjacent to the boundary behave during cell differentiation of *D. discoideum*.

Nuclei were isolated from various developmental stages and photoreacted on ice using UV irradiation for 4 h. The crosslinked DNA was purified, cut with restriction endonucleases and separated on a 1.5% agarose gel, transferred onto DBM paper and hybridized with various cloned rDNA fragments. Relative to uncrosslinked DNA, the crosslinking of a restriction fragment results in a band shift in electrophoresis. A shift in electrophoretic mobility after heavy psoralen-crosslinking of DNA was observed by Carlson et al. (1982). The shift may be due to the increased molecular weight and stiffness (Carlson et al., 1982), to the increase in length (Wiesehahn & Hearst, 1978) or to kinking of DNA (Kim et al., 1983). Since initial experiments made clear that only restriction fragments of about the same size could reasonably be compared with one another in the assay system, we chose an Eco RI-Sac I double digest to generate fragments of about 2 kb in the rDNA region concerned. With this restriction digest we hoped to distinguish between the highly crosslinked region upstream from the coding region and the more distant spacer (Sogo et al. 1984). As controls, nuclei of vegetative and slug cells were treated in the same way as the other samples but no psoralen was added. Furthermore, naked DNA was crosslinked to ascertain that no specific physical properties of any given restriction fragment would prevent its comparison with the other

fragments in the electrophoretic band shift assay. Since heavy crosslinking interferes with restriction endonuclease digestion (Carlson et al., 1982), the naked DNA was restricted prior to the photoreaction.

Fig. 7 shows the accessibility of the DNA to psoralen in different chromatin regions (denoted α , β , γ and δ in Fig. 7D) during cell differentiation. Naked AX-2 DNA restriction digests confirm the mapping of the cloned fragments (Fig. 7, lanes 1 and 2). Eco RI-Sac I double digests are shown (Fig. 7, lanes 3 to 10). Uncrosslinked controls from vegetative (track 3) and slug (track 9) nuclei show the maximum (=normal), heavily crosslinked naked DNA (track 10) the minimum (=maximal shift) electrophoretic mobility a given restriction fragment can exhibit in the assay system. To demonstrate that the shift in electrophoretic mobility is not due to variations in the amounts of DNA, we have applied to track 8 five times the amount of DNA run in track 4. The various developmental stages of *D. discoideum* in the crosslinking experiments with isolated nuclei are indicated (Fig. 7, lanes 4 to 8).

3.2.1.1 5' NON-TRANSCRIBED SPACER REGION

Fig. 7A presents an examination of the chromatin structure of a 2.2 kb Sac I-Eco RI fragment (α) located 2.6 kb upstream from the rRNA initiation site. Crosslinking the fragment in nuclei results in a small electrophoretic shift as compared with uncrosslinked DNA, thus indicating the limited accessibility of DNA in chromatin, psoralen presumably reacting with the linker region between nucleosomes (Sogo et al., 1984). The considerable decrease in the electrophoretic mobility of heavily crosslinked naked DNA clearly demonstrates that the DNA is highly protected in chromatin. No significant changes in the accessibility of chromatin to psoralen accompany cell differentiation in this region.

Fig. 7B shows a hybridization with pEPv, thus allowing us to examine the chromatin structure of the 2.6 kb Eco RI-Sac I fragment (β) immediately upstream from the coding region. The preceding hybridization with pSE (Fig. 7A) was not stripped from the DBM paper and therefore the bands in Fig. 7A are also present in Fig. 7B. In the chromatin of all cell differentiation stages analyzed, an increased crosslinking efficiency as compared to the more upstream region is obvious (i.e. the band drift is greater). This effect results from differences in the chromatin structure of this region and is not due to the base composition of the restriction fragment, since the crosslinked naked DNA actually incorporates rather less psoralen than the 2.2 kb fragment (α) shown in the hybridization with pSE. A small but significant gradual decrease in the electrophoretic mobility of the DNA crosslinked in chromatin of differentiating cells, as compared with vegetative cells, points to structural changes in this region during development. The extent of DNA crosslinking in nuclei of 12 h and 16 h differentiated cells is still higher than in the region 2.6 kb upstream from the initiation site (fragment (α , see Fig. 7A for comparison).

3.2.1.2 CODING REGION

Fig. 7C shows the effects of psoralen-crosslinking on a 2.6 kb fragment (δ) in the coding region of the rDNA. The same blot was used as in Figs. 6A and 6B, but it was stripped prior to hybridization with pEcoVII. The structural organisation in the coding region is already well characterized. Highly active vegetative cells contain rows of transcription complexes so tightly packed that nucleosomes apparently cannot form between them (Ness et al., 1983; Labhart et al., 1983; Sogo et al., 1984). In differentiating slug cells, the transcription unit reverts to a nucleosomal arrangement (Ness et al., 1983). The psoralen-crosslinking experiment reflects these known structural features. The DNA in isolated nuclei of vegetative cells is highly accessible to psoralen. This is due either to the absence of most of the histones or to a loose histone-DNA interaction, which allows full accessibility of the DNA. The transcription complexes apparently do not prevent the DNA from being crosslinked. This observation has already been made by Sogo et al. (1984). After 2 h of development, the psoralen-crosslinking efficiency diminishes. A further decrease is observed at the 12 h and 16 h developmental stages which show about the same amount of psoralen incorporation. Since the 2 h developmental stage reveals an electrophoretic mobility midway between the respective mobilities of vegetative and 12 h (16 h) differentiated cells, one can postulate that not just two distinct structural organizations, nucleosomal and nucleosome-free, are occurring, but that gradual changes take place. Furthermore, it is interesting to note that a second, less crosslinked band of the same restriction fragment weakly hybridizes with pEcoVII in vegetative and 2 h differentiated cells (Fig. 6C, lanes 4, 5 and 8). This can be explained in two ways. Firstly, a small fraction of rRNA genes within one cell could be transcriptionally inactive. Since the electrophoretic mobility of the second band is the same as the one of crosslinked DNA in nuclei of 12 h and 16 h differentiated cells, we then can assume that these inactive genes are packed into nucleosomes. Secondly, it is possible that the less crosslinked band represents rDNA of cells engaged in cell division, a process which affords a high condensation of chromatin. If the second assumption is true, cell division has not stopped completely after 2 h of development.

Hybridization with pMG6 - which shows the effect of psoralen-crosslinking in another fragment (2.2 kb Eco RI-Sac I, γ) within the coding region - gave the same results as hybridization with pEcoVII (Eco RI-Sac I fragment δ) (results not shown).

Occasionally, some hybridization bands can be seen which run on gels above the maximum drift expected for psoralen-crosslinking (Fig. 7A, B and C; e.g. lane 8). These bands arise due to incomplete digestion of the DNA after psoralen-crosslinking (Carlson et al., 1982) and can be identified by reference to the restriction map (see Fig. 1). For instance, it has been confirmed that the weakly hybridizing band marked by an arrow (Fig. 7B, track 8) hybridizes with pPS, thus proving that the restriction endonuclease cleavage of the Sac I site -23 bp upstream from the transcription initiation point is affected by crosslinking with psoralen (results not shown).

Supercoiling of DNA influences psoralen binding (Sinden et al., 1980) and it could be that a

difference in supercoiling between active and inactive chromatin contributes to the difference in crosslinking observed. Supercoiling of transcribed DNA *in vivo* has been repeatedly postulated (for a review, see Lilley, 1983). We do not think that in our experiments psoralen binding is influenced by supercoiling. Firstly, the high endogenous nuclease activity present in our nuclei isolations (see 3.2.2) would probably lead to relaxation of a torsionally stressed chromatin conformation. Secondly, Novobiocin did not have any effect in the crosslinking experiments. Novobiocin acts by blocking topoisomerase II-generated torsional stress in active chromatin (Villeponteau et al., 1984).

In summary, we draw two major conclusions from the psoralen-crosslinking experiments. Firstly, chromatin possesses at least two distinct organizations, as far as their accessibility to psoralen-crosslinking is concerned. The boundary between these two domains appears to be located about 2.5 kb 5' to the initiation site of transcription, an estimation which is in agreement with the previous electron microscopic data obtained from rDNA of vegetative cells (Sogo et al., 1984). Moreover, our assay system indicates that the 5' structural boundary persists throughout cell differentiation. Secondly, the 5' non-transcribed spacer region immediately upstream from the transcription unit as well as the coding region itself undergo gradual changes in chromatin structure during the progressive shut-down of rRNA synthesis. The chromatin structure of the non-transcribed spacer region further upstream from the rRNA gene does not appear to change during differentiation.

3.2.2 CHROMATIN STRUCTURE ANALYSED BY INDIRECT END-LABELLING

The psoralen-crosslinking experiments permit recognition of distinct chromatin organizations and the detection of structural changes accompanying cell differentiation. They provide no information about local structural differences, such as hypersensitive sites (HSS) (Elgin, 1981; 1984). We were therefore interested in a fine structural mapping of the rDNA in the 5' non-transcribed as well as in the coding region. In particular, we wished to map the 5' structural boundary. We chose the method of indirect end-labelling first applied by Nedospasov and Georgiev (1980) and by Wu (1980) to obtain the additional structural data.

Isolated nuclei from vegetative and 16 h differentiated slug cells were mildly digested for 45 s and 2 min with micrococcal nuclease (MNase) or DNase I at 25°C. To monitor the endogenous nuclease activity present in our isolations, nuclei were autodigested at the same temperature. Incubation was for various times up to 20 min; however, no significant changes in digestion patterns were obtained after 2 min. A band pattern resembling exogenous nuclease digestion is obtained, although the bands are generally weaker. In all indirect end-labelling experiments, naked DNA controls digested at 0°C were included because of the known sequence-specificity of MNase

(Dingwall et al., 1981; Hörz and Altenburger, 1981). Though we tried several procedures to isolate "instant" DNA (see 2.5), we never succeeded in obtaining totally uncleaved DNA. Hence, we show in every indirect end-labelling experiment an overloaded track with purified "instant" DNA prior to its MNase and DNase I control digestion. These samples clearly demonstrate that the weak residual endogenous nuclease activity present in our "instant" DNA preparations does not significantly affect the cleavage pattern of the naked DNA control digestions. Markers were the 123 bp BRL ladder and Eco RI-Hind III double digested λ DNA standards. The BRL ladder was cohybridized with the rDNA samples. Occasionally we ran partial restriction endonuclease digests beside the chromatin samples to provide an endogenous marker for a precise mapping of the chromatin structure. We always included nuclear AX-2 DNA double restriction endonuclease digests which cleaved out the genomic fragment complementary to the hybridization probe. Firstly, this enabled us to visualize the DNA region of the end-labelling probe in the electrophoretic separation and, secondly, to monitor the washing conditions of the blots to minimize unspecific hybridizations. All DNA samples were cleaved to completion with the appropriate restriction enzyme, separated on a 1.5% agarose gel and transferred onto DBM paper. Hybridization was performed with a short ^{32}P -labelled DNA fragment specific for one end within the larger restricted genomic DNA fragment of interest. The partial cleavage products, which extended from the chosen restriction site carrying the sequence complementary to the hybridization probe, could then be visualized. The largest fragment was the uncleaved genomic restriction fragment. The sizes of smaller fragments gave the positions of the nuclease cleavage sites relative to the end-labelled restriction site. When applying the indirect end-labelling technique, it is important to use small and clean hybridization probes, otherwise the cleavage pattern becomes unclear. For this reason we have cloned a number of ~200 bp restriction rDNA fragments suitable for analyzing the 5' non-transcribed and the coding regions. All samples were run on the same gel; the MNase and DNase I chromatin digestion times were identical for all indirect end-labelling experiments.

3.2.2.1 5' NON-TRANSCRIBED SPACER REGION

We have used pPvH, pHPv and pSB to examine the 5' non-transcribed spacer region by the indirect end-labelling technique (see Fig. 1).

Fig. 8 shows an indirect end-labelling starting from the Bgl II site (+550 bp) within the ETS and extending into the 5' upstream region. The first three tracks show the 123 bp ladder marker (Fig. 8, lane 3) and partial restriction endonuclease cleaved AX-2 naked rDNA (Fig. 8, lanes 1 and 2). The Sac I-Bgl II double restriction endonuclease digest cleaves out the genomic rDNA fragment complementary to the pSB insert used as a hybridization probe (Fig. 8, lane 4). Strong MNase and DNase I HSS (HSS1, HSS2 and HSS3) occur in differentiated slug cells (Fig 8, lanes 11, 12, 18 and 19), where the rDNA synthesis is considerably reduced. Hypersensitive sites are more sensitive by an order of magnitude than active (or potentially active) gene regions and greater than two

orders of magnitude more sensitive than inactive chromatin regions (Wu et al., 1979). Hypersensitivity in chromatin generally involves short regions of approximately 50-400 base pairs and these have mostly been localized to the region near the 5' end of actively transcribed genes (Elgin, 1981, 1984). No nuclease hypersensitive sites in this context are observed in vegetative cells (Fig. 8, lanes 8, 9, 15 and 16) where maximum rRNA gene activity occurs. However, the entire region framed by HSS1 and HSS3 in slug cells is very nuclease-sensitive in vegetative cells. To obtain a more precise mapping of the HSS, we mixed the partial restriction endonuclease cleaved AX-2 naked rDNA samples (Fig. 8, lanes 1 and 2) with the MNase and DNase I digests (data not shown); HSS1, HSS2 and HSS3 are located at ~200, ~1700 and ~2300 bp from the initiation site of transcription (+1 bp). HSS1 is located around the two Ava II sites at -159 and -294 bp. This rDNA region has been sequenced by Hoshikawa et al. (1983). At -250 to -120 bp they found a thrice repeated sequence compassing 16 nucleotides ($A_A^T TTT_C TCTAAGTTA_G^T$), in which there were mismatches of three or less nucleotides. Such repeated sequences have also been found near the transcriptional initiation site of rRNA genes in other eukaryotes (for references see Hoshikawa et al., 1983). Recently, Bonven et al. (1985) showed that these sequences were part of a site-specific topoisomerase I consensus sequence ($A_A^G A C T T A G_A G A A A T T T_A A A$) found in *Tetrahymena*. Direct and inverted repeats of these sequences are found at -129, -169, -208, -233 and -270 bp from the start site of transcription in *D. discoideum*. More recently, Ness et al. (1986) have shown topoisomerase-like activity (SDS-inducible cleavage) in *D. discoideum* rDNA. A comparison between the mapped HSS and the sites cleaved after the addition of SDS in the 5' flanking region of the rRNA gene found by Ness et al. (1986) is given below (see 3.3).

In addition to the HSS, differences between the nuclease cleavage pattern of vegetative (Fig. 8, lanes 8, 9, 15 and 16) and slug cell nuclei (Fig. 8, lanes 11, 12, 18 and 19) occur in the proximity of the rRNA initiation site. The most prominent stretch of DNA which is cleaved in differentiated slug but not in vegetative cells is marked by an arrowhead and a circle. The selection of probe and restriction endonuclease allows a precise mapping of this stretch of DNA to sequences located at the transcription start site. Comparative data for eukaryotic rRNA genes suggest an important role in transcription control for sequences located within the vicinity of the initiation site (Sommerville, 1984). We have, moreover, noticed that the glucocorticoid receptor DNA binding sequence (5'-T-G-T-T-C-T-3'; Scheidereit and Beato, 1984) occurs precisely at the rRNA initiation site in *D. discoideum*. It is possible that the differences between vegetative and slug cells in this rDNA region occur because of the interaction of (a) trans-acting factor(s).

Very obvious is a sharp change of the overall nuclease-sensitivity both of MNase and DNase I in the 5' flanking region of the rRNA genes at about -2300 bp in the region of HSS3 (Fig. 8). Taking into account the electron microscopic data supplied by Sogo et al. (1984) and our own psoralen-crosslinking experiments described above (see 3.2.1), we conclude that the 5' structural boundary of the active nuclease-sensitive rDNA chromatin domain is located ~2.3 kb upstream

from the transcription-start site. This boundary persists, regardless of the actual transcription rate of the rRNA genes. However, in the proximity of the boundary, changes in the nuclease-cleavage pattern do take place when the rRNA synthesis has decreased (Fig. 8, e.g. compare lanes 8 and 9 with lanes 11 and 12). Besides the HSS in this rDNA region in slug cell nuclei mentioned above, minor differences distal (upstream) from the boundary are apparent when compared with vegetative cell samples.

As can be seen from the naked nuclear DNA digest (Fig. 8, lane 6), MNase displays a strong DNA sequence-specificity. Up to the 5' boundary at -2.3 kb discussed above, the majority of MNase sites cleaved in naked DNA are also present in chromatin (Fig. 8, lane 6 compare with lane 8, 9, 11 and 12). We conclude that either the DNA in this region is not protected, e.g. by nucleosomes, or that the nucleosomes are organized randomly or in many different phases. The MNase cleavage sites at the HSS are also detected following digestion of naked DNA. However, only a region which appears to coincide with HSS3 is strongly cleaved by MNase in naked DNA. This cleavage region is analysed in more detail in Fig. 9. DNase I displays much less sequence-specificity; the naked DNA digests gave only weak bands imposed on a smear (Fig. 8, lanes 13). The slug chromatin DNase I HSS (Fig. 8, lanes 18 and 19) do not appear in the naked DNA digests. We conclude that the HSS1, 2 and 3 are not an artefact of the sequence-specificity of the nucleases used, but are due to the packaging of the slug cell DNA into chromatin.

The endogenous nuclease activity in the nuclei isolations was determined by an incubation for 20 min at 25⁰ C (Fig. 8, lanes 7, 10, 14 and 17). The cleavage sites are identical with the ones obtained after exogenous nuclease digestions, although the bands are generally weaker. The cleavage region which coincides with HSS3 is strongly digested.

We wished to map more precisely the 5' structural boundary region identified in the experiment described in Fig. 8. The pHpV plasmid was chosen, thus permitting end-labelling from the -1530 bp Pvu II site upstream towards the palindrome centre (Fig. 9). The position of the Eco RI site between Eco RI fragments II and V (see Fig. 1A) relative to the MNase and DNase I cleavage fragments is shown in the Pvu II-Eco RI double digest (Fig. 9, lane 3). The Pvu II-Hpa II double digest (Fig. 9, lane 2) cleaves out the genomic AX-2 rDNA fragment complementary to the hybridization probe. The 123 bp BRL ladder is also shown (Fig. 9, lane 1). As already known from the indirect end-labelling experiment of Fig. 8, the susceptibility to either of the nucleases, but especially to MNase (Fig. 9, lanes 8, 9, 10 and 11), is sharply reduced in the rDNA region upstream from -2350 bp. This is not due to DNA polymorphism (Udvardy and Schedl, 1983), since naked DNA (Fig. 9, lane 5) undergoes no such abrupt change in the frequency of nuclease cutting within the same rDNA region (see Fig. 8 for an overview). Besides the sharp change in nuclease accessibility of chromatin DNA, the boundary at -2350 bp (thick arrow) is defined by a second property. This becomes evident when the MNase cleavage patterns of naked (Fig. 9, lane 5) and chromatin DNA (Fig. 9, lanes 7, 8, 10 and 11) are compared. From the Sac I site at +329 bp up to

the 5' boundary at -2350 bp, all major chromatin DNA cleavage sites are also cleaved in naked DNA (Fig. 8). This is the case both for the region between the boundary and the transcription start site (Fig. 8 and 9) and for the coding region (see below and Fig. 11, 12 and 13). In the rDNA spacer region (more upstream than the 5' boundary; indicated by an arrow), the naked DNA cleavage sites always occur in between the chromatin DNA cleavage sites (Fig. 9).

The boundary at -2350 bp also defines the upstream border of the HSS3 (Fig. 9).

Differences in the MNase cleavage patterns between vegetative and slug cell nuclei were observed (Fig. 9, compare lanes 7, 8 with 10, 11). In particular, two bands at -2950 and -2600 bp appeared in slug cells (Fig. 9 lanes 10, 11). These bands were also obtained by endogenous nuclease treatment in slug but not in vegetative cell samples (Fig. 9, compare lanes 6 and 9). On the other hand, DNase I digestion revealed no significant differentiation between vegetative and slug cell samples (Fig. 9, compare lanes 15, 16 and 18, 19). Close examination of the autoradiograms indicated that the bands at -2950 and -2600 bp were not present after DNase I treatment (a distinction not possible to make on the photographs). The differences seen between the two enzymes DNase I and MNase reflect differences in the cutting properties of the nucleases. DNase I, for example, recognizes features of the internal organization of nucleosomes. MNase, on the other hand, differentiates between nucleosome core and linker regions and may recognize differences related to the higher orders of structure.

Fig. 10 shows indirect end-labelling of the same rDNA region as the one described in Fig. 9. Nuclease digested and subsequently purified DNA was again cleaved to completion with Pvu II. However, by hybridizing to pPvH, the (distal) Pvu II site at -5730 bp instead of the (proximal) one at -1530 bp was labelled. Lane 1 is the 123 bp BRL ladder. The genomic rDNA fragment complementary to the hybridization probe is cleaved out by the Pvu II-Hpa II double digest (Fig. 10, lane 2). When the naked DNA nuclease digests (Fig. 10, lanes 4 and 11) are compared with the corresponding chromatin DNA digests (Fig. 10, lanes 6, 7, 9 and 10 for MNase; lanes 13, 14, 16 and 17 for DNase I), the alternating cleavage pattern predicted for this rDNA region (based on hybridization with pHPv, shown in Fig. 9) becomes very clear. The sequences cleaved most readily in naked DNA appear to be most protected in chromatin. DNase I and MNase give the same results in this respect. The rDNA region extending from -3950 to -5730 is highly resolved by electrophoresis and reveals MNase and DNase I cleavage sites 180 or 350 bp apart. This suggests a nucleosomal organization of the chromatin. Vegetative and slug cell chromatin show similar cleavage patterns, although minor differences are evident with both nucleases in the region between -4150 and -4750 bp. Especially in the DNase I digests, additional cleavage sites occur in slug (Fig. 10, lanes 16 and 17) versus vegetative (Fig. 10, lanes 13 and 14) cell chromatin.

The electron microscopic data obtained from psoralen-crosslinked vegetative cell nuclei (Sogo et al. 1984) indicate a 0.8 kb stretch of strongly crosslinked (accessible) chromatin about 2.8 kb from the -2.3 kb boundary. Such regions were found in 30 to 40% of all the molecules analysed by

electron microscopy. This suggested that the 0.8 kb DNA region resembles the highly crosslinked coding region. Hence, the former region should be located some 5.0 to 6.0 kb upstream from the rRNA initiation site. This rDNA region is highly resolved in the indirect end-labelling experiment shown in Fig. 10. However, the cleavage sites in naked and chromatin DNA alternate in the typical way described and no nuclease cleavage pattern comparable with the coding region (see below) is found. The 0.8 kb region at -4150 to -4750 bp, which shows differences between vegetative and slug cells in the nuclease digestions, is probably not located far enough upstream from the transcription unit to be assigned to the highly crosslinked stretch of rDNA identified by electron microscopy.

Summarizing, the 5' structural boundary predicted by electron microscopy (Sogo et al., 1984) and by our own psoralen-crosslinking experiments (see 3.2.1) could be precisely determined. We have demonstrated that the boundary persists throughout cell differentiation, regardless of the transcriptional activity of the rRNA genes. Indirect end-labelling maps the boundary 2.3 kb from the start site of transcription, defined by a sharp change in the nuclease-sensitivity of chromatin DNA. Furthermore, from the boundary towards the palindrome center, the nuclease cleavage sites in naked DNA are always positioned between the sites in chromatin DNA. However, in the 5' non-transcribed spacer region extending from the boundary to the transcription unit, the nuclease cleavage sites in naked and chromatin DNA are the same. In slug cells (where rRNA synthesis is greatly reduced) three DNase I/MNase HSS occur 0.2, 1.7 and 2.3 kb upstream from the initiation site of transcription. Hence, the boundary at -2.3 kb is closely associated with a site of nuclease hypersensitivity in slug cells.

3.2.2.2 CODING REGION

We have used pSaB, pTE and pSB to examine the coding region of the rRNA genes by the indirect end-labelling method.

Fig. 11 shows an indirect end-labelling starting from the Sal I site (+1823) within the 17 S transcription unit and extending towards the 3' end of the gene. The Bgl II-Sal I restriction endonuclease double digest of nuclear AX-2 DNA cleaves out the rDNA fragment complementary to the hybridization probe; the Bgl II site at +3692 bp appears because of an incomplete digestion with this enzyme (Fig. 11, lane 1). The 123 bp ladder was used as size marker (Fig. 11, lane 2). In naked DNA digests (Fig. 11, lanes 4 and 11) similar cleavages occur as in chromatin DNA (Fig. 11, lanes 6, 7, 9, 10, 13, 14, 16 and 17). This can best be judged from the MNase samples (Fig. 11, lanes 4, 6, 7, 9, 10) which give sharper bands than DNase I (Fig. 11, lanes 11, 13, 14, 16 and 17) because of the well-known sequence-specificity of MNase (Dingwall et al., 1981; Hörz and Altenburger, 1981). Some small differences do occur between naked DNA (Fig. 11, lane 4) and chromatin DNA from vegetative cells (Fig. 11, lanes 6, 7, 9 and 10). In slug cells, the cleavage

pattern of chromatin DNA (Fig. 11 lanes 9, 10, 16 and 17) is essentially the same as that of naked DNA (Fig. 11, lanes 4 and 11). The internal transcribed sequences ITS 1 and 2 do not exhibit a characteristic cleavage pattern.

In the experiment shown in Fig. 12, end-labelling commences at the Eco RI site (+2446 bp) in the 17S transcription unit and extends to the Eco RI site (-2730 bp) in the 5' NTS. The genomic 206 bp Eco RI-Taq I fragment was cloned and used as probe in this experiment (Fig. 12, lane 1). The 123 bp BRL ladder is shown (Fig. 12, lane 2). Instant DNA was applied and used in the naked DNA digestions (Fig. 12, lane 3). As already observed in the 3' half of the transcription unit, the naked DNA (Fig. 12, lanes 4 and 11) and the chromatin DNA (Fig. 12, lanes 6, 7, 9, 10, 13, 14, 16 and 17) nuclease digestions give similar cleavage patterns. In the region of the boundary between the ETS and the 17S transcription unit there appears a very prominent nuclease cut in both naked and chromatin DNA. The 5' NTS is more nuclease sensitive than the coding region. This very sensitive zone begins upstream from the gene at HSS1 (see Fig. 8).

Fig. 13 shows an direct end-labelling commencing at the Sac I site (+329 bp) within the ETS and extending to the Sac I site (+5079 bp) within the 26S transcription unit. The same cloned 222 bp Sac I-Bgl II fragment (pSB) was used as shown in the experiment of Fig. 8. This rDNA fragment was cleaved out of genomic AX-2 DNA by the corresponding restriction endonucleases and hybridized to pSB (Fig. 12, lane 2). A partial Hpa II digest of pEcoV (see Fig. 1) serves as an endogenous marker (Fig. 13, lane 1). The 123 bp BRL ladder is, again, shown (Fig. 13, lane 3). No additional information about the chromatin structure of the coding region is gained. The naked DNA (Fig. 13, lanes 4 and 12) nuclease cleavage sites are the same as in chromatin DNA digestions, which apparently is a typical feature of the coding region. The high resolution in this experiment at the 5' end of the 17S transcription unit allow us to precisely map the pronounced cleavage site in naked and chromatin DNA mentioned above (Fig. 12) to the ETS-17S transcription unit boundary.

In summary, all the naked DNA nuclease cleavage sites in the coding region are also chromatin DNA cleavage sites in both vegetative and slug cell nuclease digests. The transcription complexes of vegetative cells do not seem to influence the nuclease accessibility. Since it is known that in slug cells the coding region reverts to a nucleosomal arrangement (Ness et al., 1983), we conclude that the nucleosomes are not in a highly ordered array.

3.3 PUTATIVE TOPOISOMERASE SITES OF ACTION IN rDNA

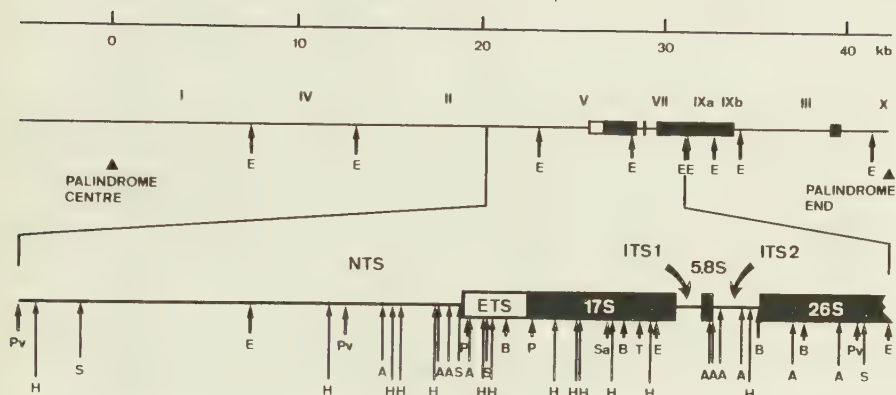
The three HSS in the 5' flanking region of the transcription unit were mapped by the indirect end-labelling technique (Fig. 8). The rDNA sequence around the HSS1 at -200 bp from the

initiation site of transcription is the only sequence so far known (Hoshikawa et al., 1983). The HSS1 occurs in a rDNA region containing inverted and direct repeats of a topoisomerase I consensus sequence previously found in *Tetrahymena* rDNA (Gocke et al., 1983; Bonven et al., 1985). It has recently been demonstrated by Ness et al. (1986) that topoisomerase-like activity occurs in the 5' flanking region of the *D. discoideum* rRNA genes. Treatment of diluted *D. discoideum* nuclei with high concentrations (1%) of SDS induces double strand cuts in the rDNA, whereas in *Tetrahymena* rDNA single strand breaks were observed. SDS aborts the normal topoisomerase reaction by denaturing the enzyme. This prevents the topoisomerase from resealing the DNA it has just cut. Topoisomerase sites of action are revealed by the cleavage sites. Topoisomerase I cuts only one strand, topoisomerase II makes double strand breaks. Treatment of isolated nuclei with 0.8 M NaCl dissociates the topoisomerase from the DNA after DNA cuts have been ligated. Hence, the subsequent addition of 1% of SDS does not reveal any cleavages.

The aim of the indirect end-labelling experiment shown in Fig. 14 was to assign the sites of topoisomerase-like activity identified in *D. discoideum* (Ness et al., 1986) to specific chromatin regions. Consequently, indirect end-labelling starting from the Bgl II site (+550 bp) within the ETS and extending into the 5' NTS was chosen. The Bgl II-Sac I double digest of AX-2 nuclear DNA cleaves out the 222 bp rDNA fragment cloned and used as a hybridization probe (Fig. 14, lane 11). Nuclei were treated with either SDS (Fig. 14, lane 9) or NaCl (Fig. 14, lane 10) as described above. To permit comparison, nuclease digested chromatin samples were run in parallel (Fig. 14, lanes 1 to 8). Vegetative (Fig. 14, lanes 1, 2, 5 and 6) and slug (Fig. 14, lanes 3, 4, 7 and 8) cell nuclei were digested with MNase (Fig. 14, lanes 2 and 4) or DNase I (Fig. 14, lanes 6 and 8). In addition, the endogenous nuclease activity present in the various isolates was determined by incubation of the nuclei for 20 min at 25°C (Fig. 14, lanes 1, 3, 5 and 7). All the samples were size-fractionated on a 1.5% agarose gel and transferred onto DBM paper prior to hybridization with pSB. This preliminary analysis enables us to assign the putative topoisomerase double-stranded cleavage sites (Fig. 14, lane 9) 0.2 and 2.3 kb upstream from the transcription unit to the HSS1 and 3 respectively. As already mentioned, HSS1 includes inverted and direct repeats of a topoisomerase I consensus sequence found in *Tetrahymena* rDNA. HSS3 is located precisely at the 5' structural boundary previously characterized.

In summary, the topoisomerase-like activity found in the 5' flanking region of *D. discoideum* maps to two of the three HSS found in the rDNA chromatin of differentiated slug cells. One of these two HSS at -0.2 kb contains inverted and direct repeats of a topoisomerase I consensus sequence, the second is located at the boundary of the active rDNA chromatin domain.

A)



B)

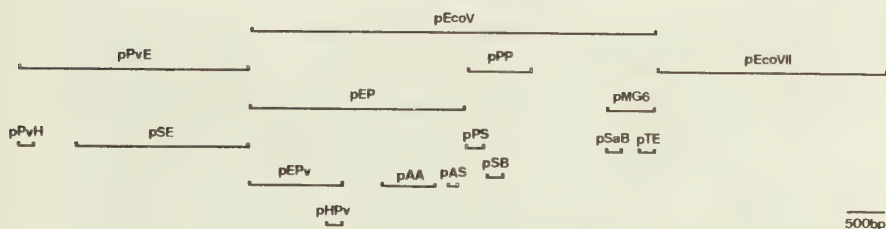


Fig. 1: (A) Restriction map of the ribosomal DNA of *D. discoideum*. The map shows half of the palindromic DNA molecule. Data are from Cockburn et al. (1976, 1978), Maizels (1976), Ozaki et al. (1984) and our own mappings (Fig. 2 and 3). The position of the 5S rRNA gene is known only approximately. The symbols for the restriction enzymes are: A, Ava II; B, Bgl II; E, Eco RI; H, Hpa II; P, Pst I; Pv, Pvu II; S, Sac I; Sa, Sal I; T, Taq I. The restriction map for Taq I and Sac I (see results) is incomplete. NTS, ETS and ITS denote the non-transcribed, external transcribed and internal transcribed spacer regions, respectively.

(B) Cloned rDNA fragments.

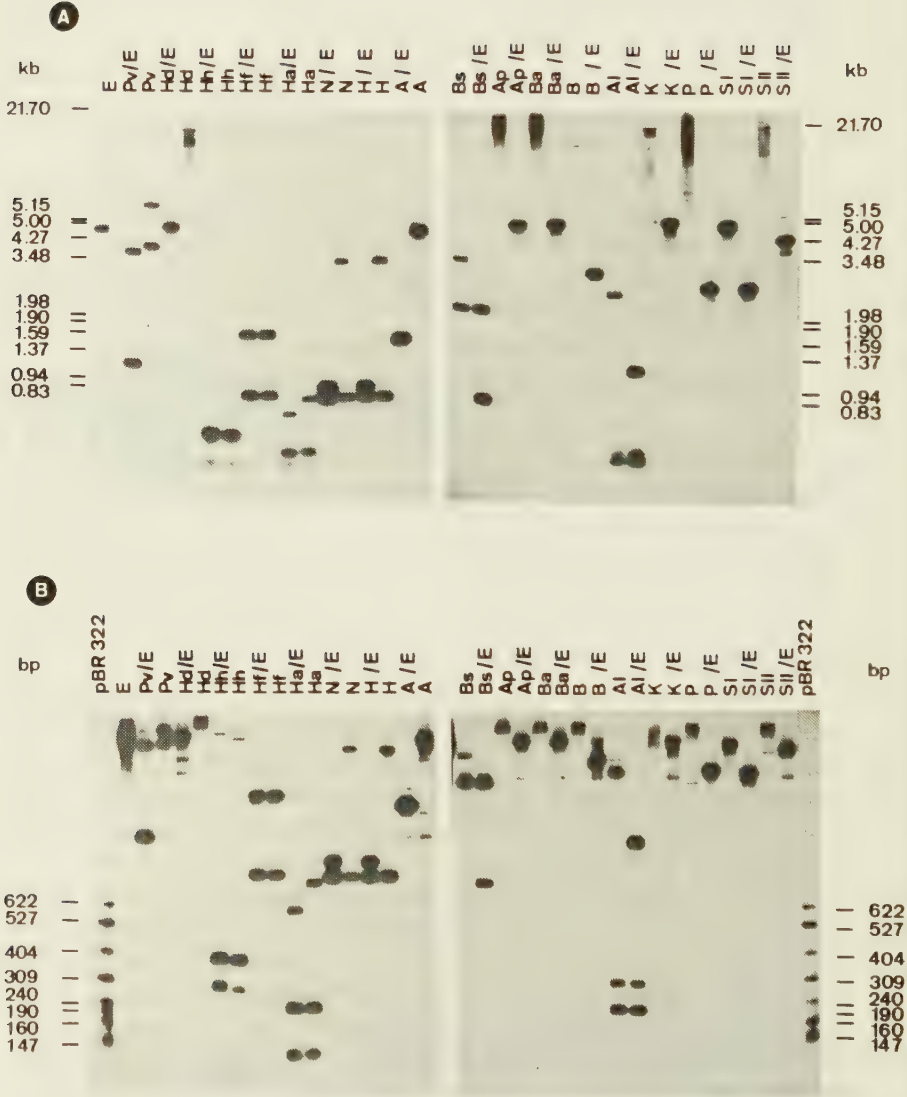


Fig. 2:

Fig. 2: Cleavage sites of restriction endonucleases in the vicinity of the Eco RI-site between Eco RI fragments II and V of the rDNA (see Fig. 1). Nucleolar rDNA was single and double digested with the indicated restriction endonucleases, size-fractionated on 0.75% (A) or 2.3% agarose gels (B), transferred onto DBM paper and hybridized with the gel-purified 1.6 kb Eco RI-Ava II fragment (derived from cloned pEcoV) upstream from the rRNA initiation site. The symbols for the restriction endonucleases are: A, Ava II; Al, Alu I; Ap, Apa I; B, Bgl II; Ba, Bam HI; Bs, Bst NI; E, Eco RI; H, Hpa II; Ha, Hae III; Hd, Hind III; Hf, Hinf I; Hh, Hha I; K, Kpn I; N, Msp I; P, Pst I; Pv, Pvu II; S I, Sac I; S II, Sac II. Markers were Eco RII-Hind III double digested λ DNA standards and Hpa II digested pBR322.

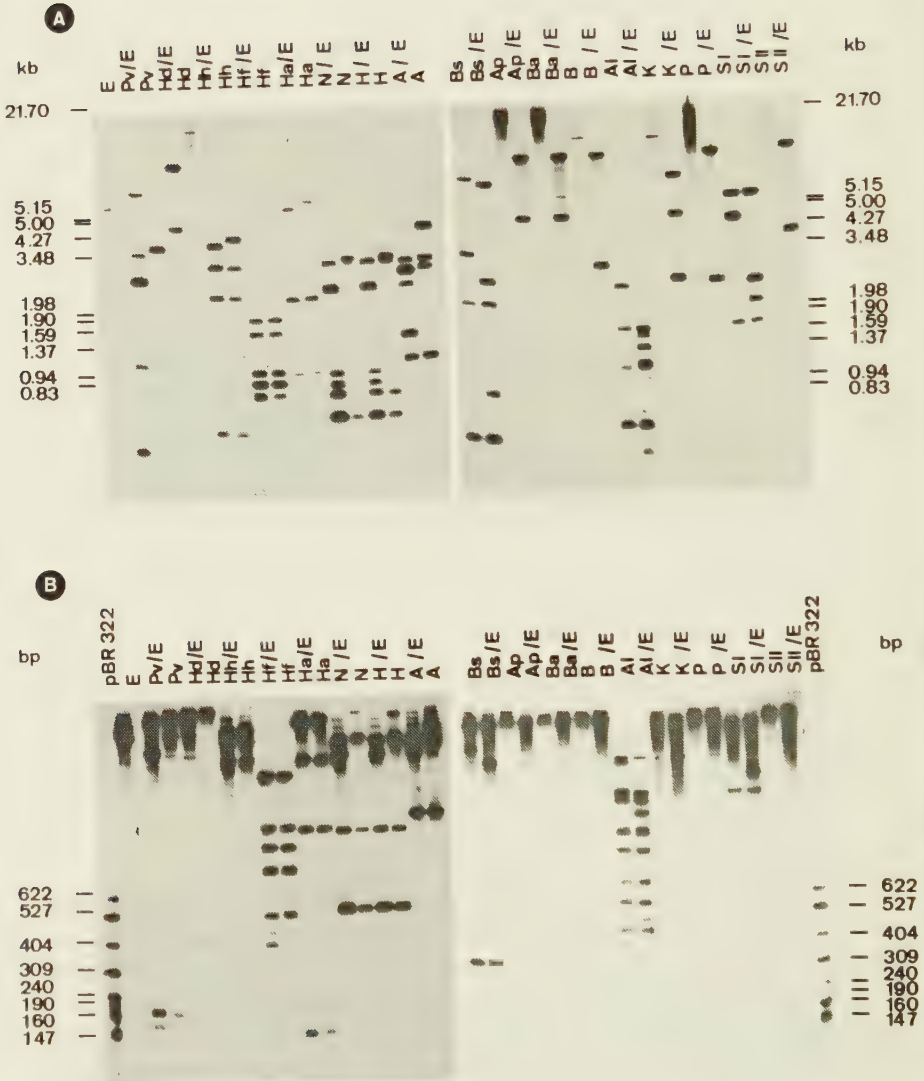


Fig. 3:

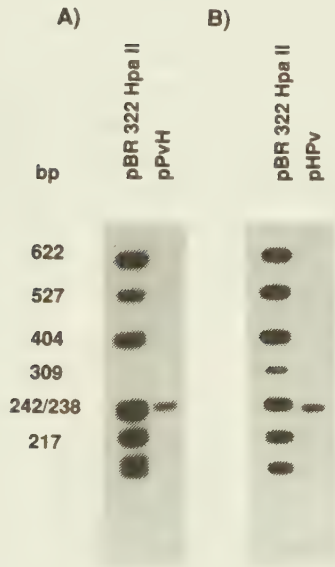


Fig. 4: Size estimation of cloned DNA fragments derived from unsequenced rDNA regions. Nuclear DNA was double digested with Pvu II and Hpa II, size-fractionated on a 4% polyacrylamide gel, transferred onto DBM paper and hybridized with pPvH (A) and pHpV (B). Marker was Hpa II digested pBR322.

Fig. 3: Cleavage sites of restriction endonucleases in the vicinity of the Eco RI site between Eco RI fragments II and V of rDNA (see Fig. 1). Nucleolar rDNA was single and double digested with the indicated restriction endonucleases, size fractionated on 0.75% (A) or 2.3% (B) agarose gels, transferred onto DBM paper and hybridized with the gel-purified Eco RI fragment II (derived from nucleolar DNA). For the symbols of the restriction endonucleases see Fig. 2. Markers were Eco RI-Hind III double digested λ DNA standards and Hpa II digested pBR322.

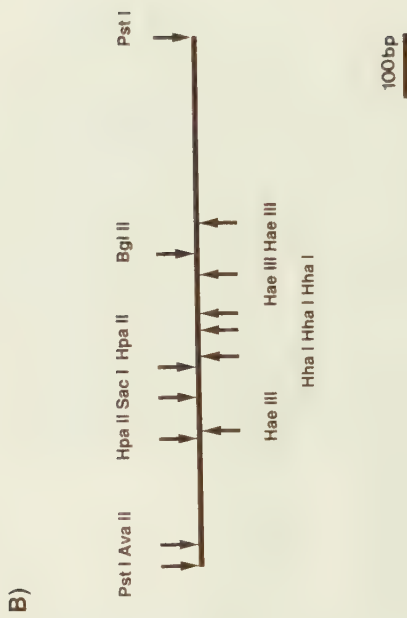
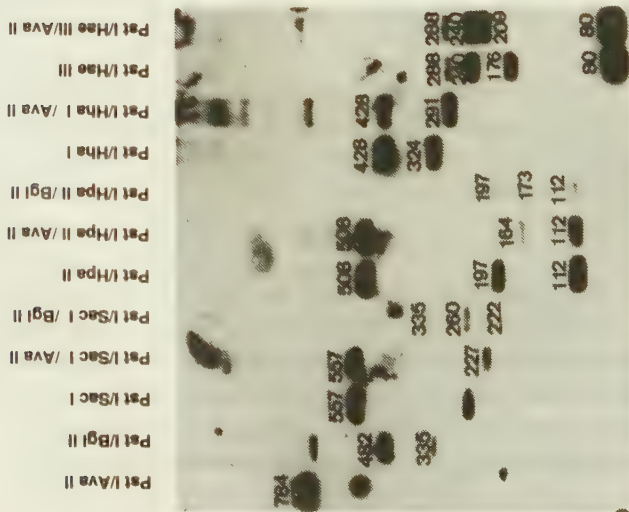


Fig. 5: (A) Analysis of the genomic DNA restriction fragments hybridizing to pPP. Nuclear *D. discoideum* strain AX-2 DNA was digested with the restriction endonucleases indicated, size-fractionated on a 4% polyacrylamide gel, transferred onto DBM paper and hybridized with pPP (see Fig. 1). The restriction fragment sizes are indicated in bp.

(B) Restriction map of the 817 bp Pst I-Pst I fragment of genomic *D. discoideum* DNA. The map is identical for *D. discoideum* strains AX-2 and A3 (Hoshikawa et al., 1983).

A) pPP



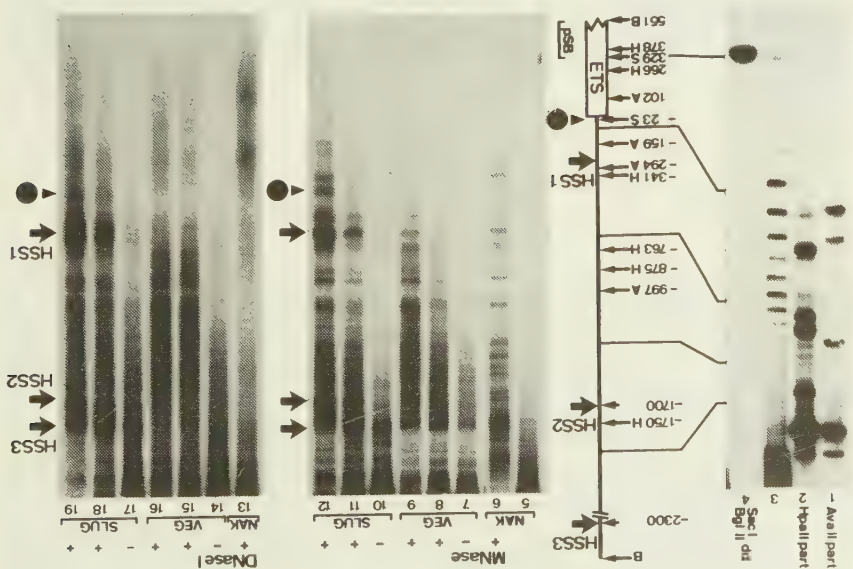


Fig. 8: Indirect end-labelling experiment. Isolated nuclei from vegetative (lanes 7 to 9 and 14 to 16) and 16 h (slug) differentiated cells (lanes 10 to 12 and 17 to 19) and naked instant nuclear DNA (lane 5) were digested with micrococcal nuclease (MN) (lanes 6, 8, 9, 11 and 12) and DNase I (lanes 13, 15, 16, 18 and 19). Lanes 7, 10, 14 and 17 show the endogenous nuclease activity. The purified DNA fragments were cut with Bgl II, separated on a 1.5% agarose gel, transferred onto DBM paper and probed with the cloned 222 bp Bgl II-Sac I fragment (PSB). Partial (lanes 1 and 2) and complete (lane 4) restriction endonuclease digests of instant DNA and the 123 bp BRL ladder (lane 3) were run in parallel. The symbols for the restriction endonucleases are: A, Ava II; B, Bgl II; H, Hpa II; S, Sac I. ETS denotes the external transcribed spacer region and HSS the nuclease hypersensitive sites, respectively. The distances from the transcription initiation site (+1) are given in bp.

Fig. 7: rDNA psoralen-crosslinking experiment. Isolated nuclei from vegetative, 2 h, 12 h, 16 h (slug) differentiated cells and naked DNA (lane 10) were crosslinked with psoralen (P) under UV irradiation. The purified DNA was double digested with Eco RI and Sac I, separated on a 1.5% agarose gel, transferred onto DBM paper and hybridized with pSE (A), pEPV (B) and pEcoVII (C). Lanes 3 and 9 show the uncrosslinked control DNA from control nuclei of vegetative (lane 3) and slug (lane 9) cells. Naked DNA (lane 10) was digested with restriction endonucleases prior to the photochemical reaction, since only partial cleavage occurred after heavy crosslinking. Crosslinking of a restriction fragment results in a decreased electrophoretic mobility as compared with non-crosslinked DNA.

(D) Restriction map and hybridization probes to the psoralen-crosslinking experiment.

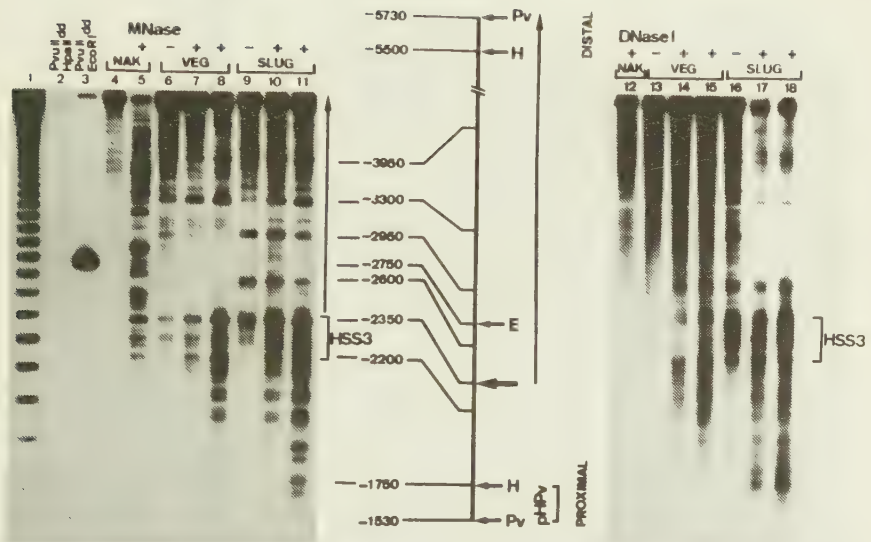


Fig. 9: Indirect end-labelling experiment. Isolated nuclei from vegetative (lanes 6 to 8 and 13 to 15) and 16 h (slug) differentiated cells (lanes 9 to 11 and 16 to 18) and naked instant DNA (lane 4) were digested with micrococcal nuclease (MN) (lanes 5, 7, 8, 10 and 11) and DNase I (lanes 12, 14, 15, 17 and 18). Lanes 6, 9, 13 and 16 show the endogenous nuclease activity. The purified DNA fragments were cut with Pvu II, separated on a 1.5% agarose gel, transferred onto DBM paper and probed with the cloned ~220 bp Hpa II-Pvu II fragment (pHPv). Restriction double digests of instant DNA (lanes 2 and 3) and the 123 bp BRL ladder (lane 1) were run in parallel. The symbols for the restriction endonucleases are: E, Eco RI; H, Hpa II; Pv, Pvu II. HSS3 denotes the hypersensitive site 3. The distances from the transcription start site (+1) are given in bp.

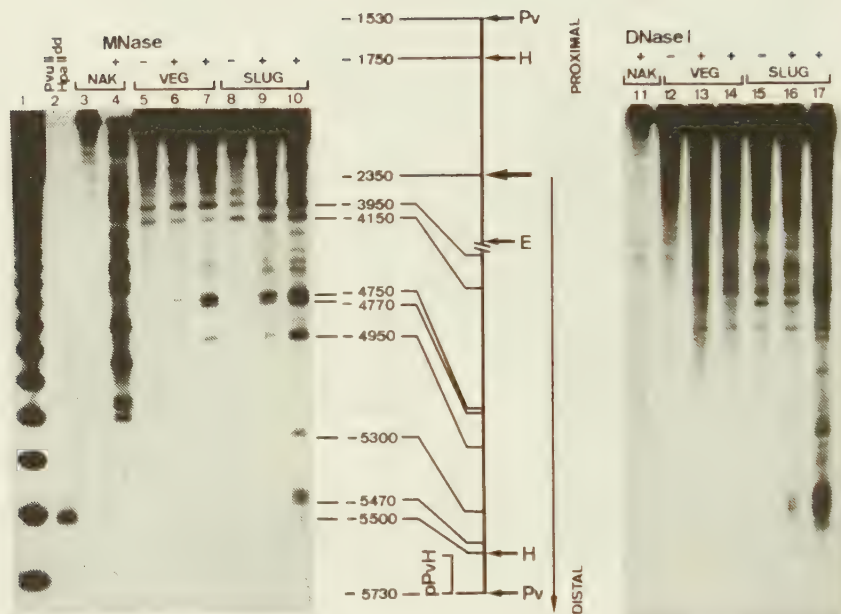


Fig. 10: Indirect end-labelling experiment. Isolated nuclei from vegetative (lanes 5 to 7 and 12 to 14) and 16 h (slug) differentiated cells (lanes 8 to 10 and 15 to 17) and naked instant DNA (lane 3) were digested with micrococcal nuclease (MN) (lanes 4, 6, 7, 9 and 10) and DNase I (lanes 11, 13, 14, 16 and 17). Lanes 5, 8, 12 and 14 show the endogenous nuclease activity. The purified DNA fragments were cut with Pvu II, separated on a 1.5% agarose gel, transferred onto DBM paper and probed with the cloned ~240 bp Pvu II-Hpa II fragment (pPvH). A Pvu II-Hpa II restriction double digest (lane 1) and the 123 bp BRL ladder (lane 2) were run in parallel. The symbols for the restriction endonucleases are: E, Eco RI; H, Hpa II; Pv, Pvu II. The distances from the transcription start site (+1) are given in bp.

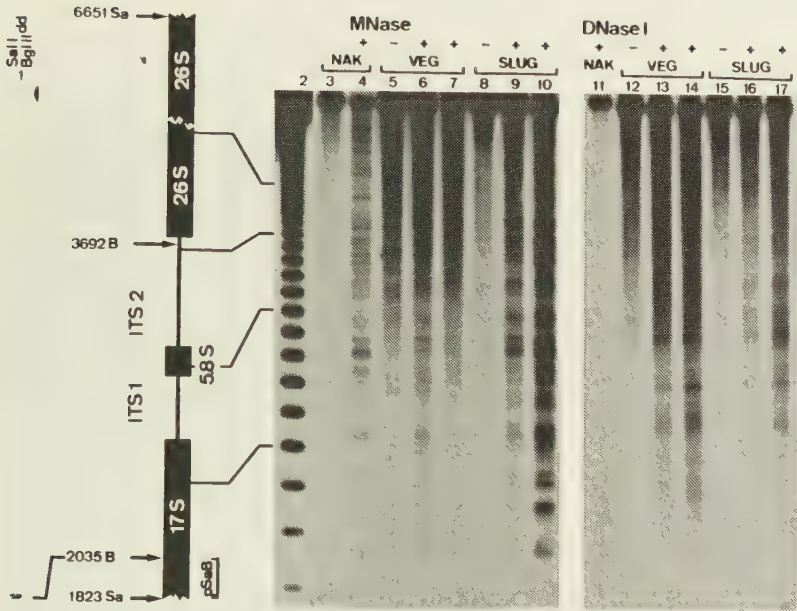


Fig. 11: Indirect end-labelling experiment. Isolated nuclei from vegetative (lanes 5 to 7 and 12 to 14) and 16 h (slug) differentiated cells (lanes 8 to 10 and 15 to 17) and naked instant DNA (lane 3) were digested with micrococcal nuclease (MN) (lanes 4, 6, 7, 9 and 10) and DNase I (lanes 11, 13, 14, 16 and 17). Lanes 6, 9, 13 and 16 show the endogenous nuclease activity. The purified DNA fragments were cut with Sal I, separated on a 1.5% agarose gel, transferred onto DBM paper and probed with the cloned 212 bp Sal-Bgl II fragment (pSaB). A restriction double digest of instant DNA (lane 1) and the 123 bp BRL ladder (lane 2) were run in parallel. The Bgl II digest in lane 1 is incomplete. The symbols for the restriction endonucleases are: B, Bgl II; S, Sal I. ITS denotes the internal transcribed spacer region. The distances from the transcription start site (+1) are given in bp.

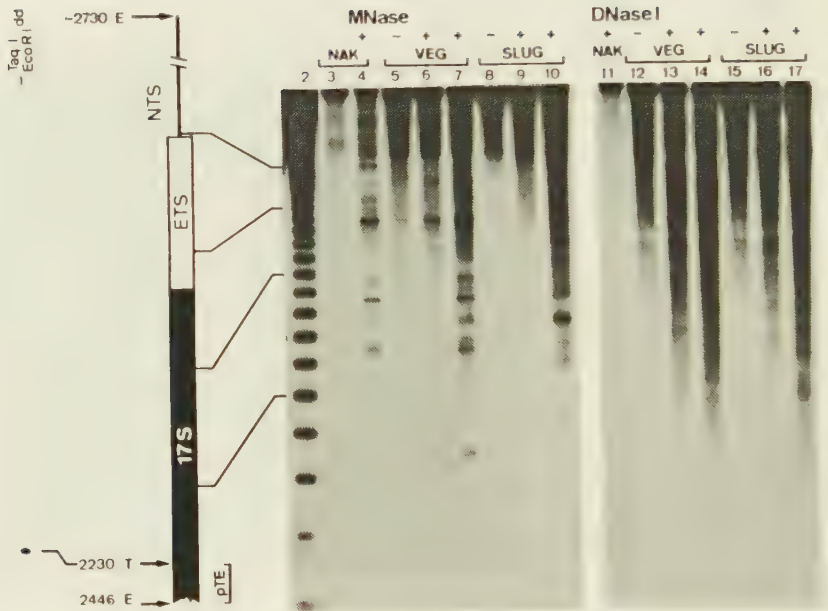


Fig. 12: Indirect end-labelling experiment. Isolated nuclei from vegetative (lanes 5 to 7 and 12 to 14) and 16 h (slug) differentiated cells (lanes 8 to 10 and 15 to 17) and naked instant DNA (lane 3) were digested with micrococcal nuclease (MN) (lanes 4, 6, 7, 9 and 10) and DNase I (lanes 11, 13, 14, 16 and 17). Lanes 5, 8, 12 and 15 show the endogenous nuclease activity. The purified DNA fragments were cut with Eco RI and probed with the cloned 206 bp Eco RI-Taq I fragment (pTE). A restriction double digest of instant DNA (lane 1) and the 123 bp BRL ladder (lane 2) were run in parallel. The symbols for the restriction endonucleases are: E, Eco RI; T, Taq I. ETS and NTS denote the external and non-transcribed spacer regions respectively. The distances from the transcription site (+1) are given in bp.

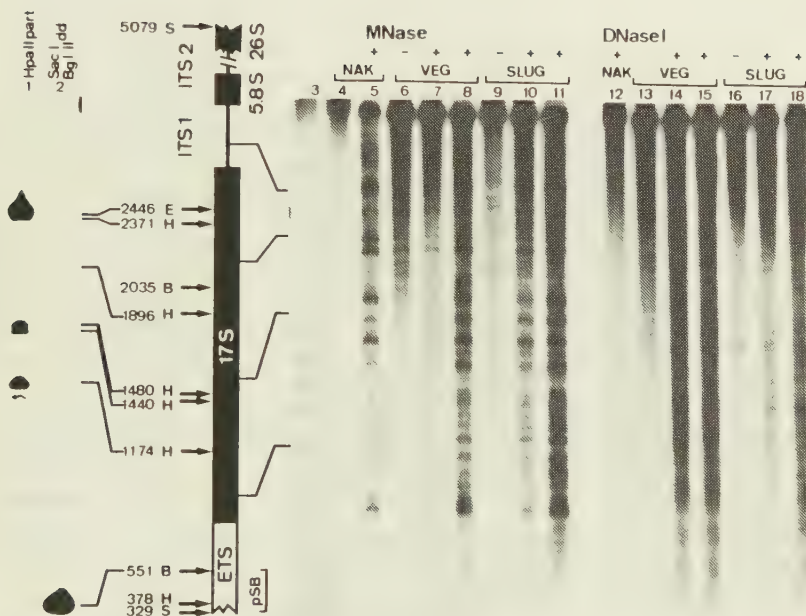


Fig. 13: Indirect end-labelling experiment. Isolated nuclei from vegetative (lanes 6 to 8 and 13 to 15) and 16 h (slug) differentiated cells (lanes 8 to 10 and 15 to 17) and naked instant DNA (lane 4) were digested with micrococcal nuclease (MN) (lanes 5, 7, 8, 10 and 11) and DNase I (lanes 12, 14, 15, 17 and 18). Lanes 6, 9, 13 and 16 show the endogenous nuclease activity. The purified DNA fragments were cut with Sac I, separated on a 1.5% agarose gel, transferred onto DBM paper and probed with the cloned 222 bp Sac I-Bgl II fragment (pSB). A partial Hpa II digest of pEcoV (lane 1), a Sac I-Bgl II double restriction endonuclease digest of instant DNA (lane 2) and the 123 bp BRL ladder (lane 3) were run in parallel. The symbols for the restriction endonucleases are: B, Bgl II; E, Eco RI; H, Hpa II; S, Sac I. ETS denotes the external transcribed spacer region. The distances from the transcription start site (+1) are given in bp.

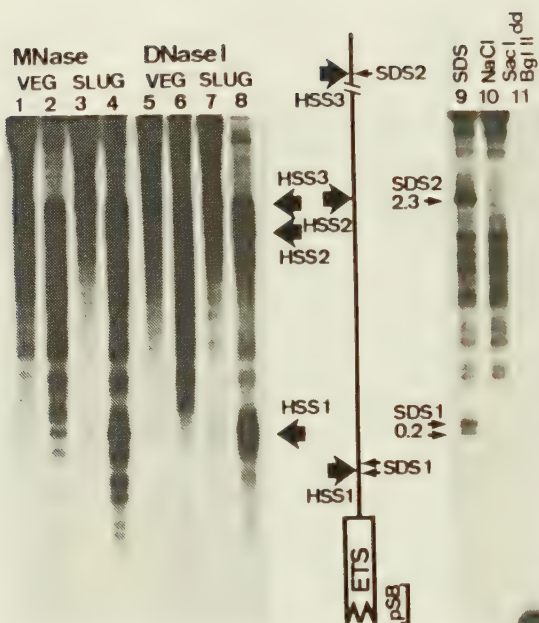


Fig. 14: SDS-induced cleavage upstream from the rRNA transcription initiation site. Vegetative nuclei were treated with NaCl (lane 10) or SDS (lane 9). DNA was extracted, digested with Bgl II and separated on a 1.5% agarose gel, blotted onto DBM paper and probed with the cloned 222 bp Bgl II-Sac I fragment (pSB). Lanes 2, 4, 6 and 8 are micrococcal nuclease (MNase) and DNase I digested samples of vegetative (V) and 16 h differentiated slug (S) cells. Lanes 1, 3, 5 and 7 show the endogenous nuclease activity of the same nuclei isolation. Lanes 1 to 8 are the same samples as in Fig. 10. Lane 11 shows a Sac I-Bgl II restriction endonuclease double digest. HSS denotes the nuclease hypersensitive sites. The distances from the transcription start site are given in kb.

4. DISCUSSION

4.1 5' STRUCTURAL BOUNDARY OF THE ACTIVE rDNA DOMAIN

4.1.1 MAPPING OF THE 5' STRUCTURAL BOUNDARY

An electrophoretic band shift assay was used to compare psoralen-crosslinked chromatin DNA in the 5' non-transcribed spacer region and the coding region of the 36 rRNA transcription unit. The experiments revealed that, with respect to the extent of psoralen-crosslinking, at least two different chromatin domains occur. The boundary between these two domains could be approximately mapped to 2.5 kb upstream from the transcription initiation point. This agreed with electron microscopic data obtained from psoralen-crosslinked chromatin DNA, which showed structural changes 2 to 3 kb upstream and downstream from the transcription unit (Sogo et al., 1984). Since it is technically impossible to isolate nucleoli from late developmental stages of *D. discoideum*, no electron microscopic data of differentiated slug cells were available. Our own psoralen-crosslinking studies indicated that the structural boundary persists during cell differentiation. By using the indirect end-labelling technique (Wu, 1980; Nedospasov and Georgiev, 1980), we mapped a structural boundary defined by a sharp change in the susceptibility to digestion by MNase and DNase I in both differentiation stages analyzed. This boundary is located 2.3 kb upstream from the rRNA transcription initiation site. We propose that, by monitoring the accessibility of the DNA in chromatin, both psoralen-crosslinking and nuclease digestion experiments recognize the same boundary.

4.1.2 STRUCTURAL CHANGES AT THE 5' BOUNDARY

4.1.2.1 NUCLEOSOME PHASING

The boundary was more precisely characterized by indirect end-labelling. A comparison of the nuclease cutting sites in chromatin of both vegetative and slug cells with those in naked DNA revealed that the cutting behaviour at the boundary changes sharply. In the nuclease-sensitive region immediately proximal to the transcription unit, there is no difference in the cleavage pattern between naked and chromatin DNA. Hence, if nucleosomes are present, they are arranged either randomly or in many different phases. A preliminary MNase analysis of the chromatin structure in this region (Schmidlin and Bettler, results not shown) reveals a nucleosomal ladder for slug cells and a smear - indicating the absence of nucleosomes - for vegetative cells. Conversely, in the nuclease-resistant region upstream from the boundary the sequences attacked most readily in naked DNA appear to be most protected in chromatin. This alternating cleavage pattern has previously

been reported in the region 3' from the 36S rRNA coding region extending to the palindrome terminus as well as in the central spacer. The term "site-specific" phasing was introduced (Edwards and Firtel, 1984; Parish et al., 1986). Although much of the site-specific phasing was irregularly spaced, Edwards and Firtel (1984) found that salt extraction data were consistent with the presence of nucleosomes. They proposed that the phasing signal and the nuclease-sensitive site in naked DNA may be centred within the nucleosome and may be identical. In the electron microscopic experiments with psoralen carried out by Sogo et al. (1984), the transcriptionally inactive bulk chromatin showed regularly spaced uncrosslinked stretches of DNA of about 200 to 400 bp, whereas the coding region was heavily crosslinked. The small uncrosslinked stretches of DNA indicated the presence of nucleosomes during crosslinking. The data by Edwards and Firtel (1984), Sogo et al. (1984) and Ness et al. (1983) are consistent with a model in which the entire transcriptionally inactive bulk chromatin of the *D. discoideum* extrachromosomal rDNA palindrome is covered with nucleosomes. We propose that in vegetative cells the structural boundary marks a change of nucleosome-free DNA (in the nuclease-sensitive 5' NTS) to the phased nucleosomes of bulk chromatin (in the nuclease-resistant 5' NTS). In differentiating cells, where rRNA synthesis is low, we propose that a change occurs from unphased nucleosomes (in the nuclease-sensitive chromatin) to the phased nucleosomes of bulk chromatin. It is not known if the more structured nucleosomal order in the bulk chromatin is due to the underlying DNA sequence (Linxweiler and Hörz, 1985) or if it is an effect of the boundary (Kornberg, 1981).

4.1.2.2 HIGHER ORDER CHROMATIN STRUCTURE

Generally, it is not clear whether nuclease-sensitivity results from nucleosomal removal or an altered conformation of individual nucleosomes. It may also involve changes in the higher orders of chromatin structure (e.g. Stalder et al., 1980b; Smith et al., 1984; Weintraub, 1984).

Although we did not perform specifically designed experiments to address this question (e.g. Smith et al., 1984), the crosslinking experiments with psoralen suggest that a switch in the folding of the nucleosomal filament does occur at the domain boundary. The electrophoretic band shift assay showed less drifting for crosslinked DNA fragments derived from bulk chromatin than for fragments derived from active (nuclease-sensitive) chromatin regions which had reverted to a nucleosomal arrangement (Ness et al., 1983; Schmidlin and Bettler, results not shown) following the strong reduction in rRNA synthesis. In this situation, both of the chromatin regions adjacent to the boundary are covered with nucleosomes. The low accessibility to psoralen in bulk chromatin can be attributed to a tighter compaction in this region than in the nuclease-sensitive slug domain. This additional compaction points to a higher organizational form. Obviously, there are also other explanations. Firstly, the density of nucleosomes in the active chromatin of slug cells could be substantially lower than in bulk chromatin. Secondly, the electrophoretic band drift is not merely a function of the overall extent of crosslinking, but also depends on the altered physical properties of

a DNA fragment after treatment with psoralen. It is possible that a high degree of local crosslinking, such as is expected at the HSS, mimics a high overall crosslinking in the electrophoretic assay. Since fragments derived from three different regions of the active domain gave identical results, this explanation is not very probable but cannot be ruled out completely. Thirdly, it may be that psoralen recognizes an altered nucleosomal conformation.

We have recently used MNase digestion of isolated differentiated slug nuclei to compare the nucleosomal ladder obtained on either side of the boundary (Schmidlin and Bettler, results not shown). In the 5' NTS proximal to the coding region, a clear nucleosomal pattern resembling the one in the coding region was obtained. The bulk chromatin - on the more upstream side of the boundary - gave a complex banding pattern, which was not due solely to sequence specific cutting by MNase. This unusual pattern probably originates in the reduced accessibility of the nucleosome linkers in a chromatin suprastructure.

The combined results suggest that the phased nucleosomes of bulk chromatin are organized into a higher order of chromatin structure whereas the unphased nucleosomes of the coding region and its neighbouring sequences are not. We postulate that folding of the nucleosomal filament into a supranucleosomal conformation commences at the 5' boundary.

4.2 TOPOISOMERASE-LIKE ACTIVITY IN THE 5' FLANKING REGION OF THE rRNA GENE

Although most of the eukaryotic genome is not under torsional stress (Sinden et al., 1980), recent data circumstantially suggest that supercoiling is required for effective transcription of pol I/pol III (Glikin et al., 1984; Ryoji and Worcel, 1984; Pruitt and Reeder, 1984; Kmiec and Worcel, 1985)) and pol II (Lilley, 1984; Villeponteau et al., 1984; Han et al., 1985) genes (see also North, 1985). The enzymes that mediate topological changes are the topoisomerases (Reviewed by Wang, 1985). Some decondensations of the chromatin superstructure probably occurs in the transcriptionally active regions (Pelling, 1964; Simon et al., 1985; Daneholt et al., 1982; Weintraub and Groudine, 1976) and topoisomerase activity is presumably required for this decondensation. Topoisomerase I is preferentially associated with transcriptionally active genes, including nucleolar genes (Weisbrod, 1982; Weintraub, 1985; Fleischmann et al., 1984; Muller et al., 1985; Bonven et al., 1985; Higashikawa et al., 1977). Gilmour et al. (1986) showed that topoisomerase I is associated with the transcribed regions and proposed that this topoisomerase allows topological changes in DNA that are required for transcription. Topoisomerase II is a major structural element of both interphase nuclei and mitotic chromosomes (Berrios et al., 1985; Earnshaw et al., 1985) and may be associated with specific sequences (Cook and Brazell, 1980; Nelkin et al., 1980) at the bases of the loops of mitotic chromosomes (Earnshaw and Heck, 1985). This suggests it has a functional role in regulating the superhelical strain in chromatin domains. Regions of loop anchorage shown in nontranscribed spacer sequences of *Drosophila* gene repeats (Mirkowitch et

al., 1984) are major topoisomerase II cleavage sites in naked DNA in vitro (Udvardy et al., 1985). Characterization of a scaffold attachment site in the *Drosophila* histone gene cluster revealed an enrichment for sequences closely related to the topoisomerase II cleavage consensus in this region (Gasser and Laemmli, 1986). Moreover, it was recently demonstrated that the matrix association region of the kappa immunoglobulin gene occurs next to an enhancer in a region containing topoisomerase II sites (Cockerill and Garrard, 1986). According to Villeponteau et al. (1984), nuclease-sensitivity reflects a change in the superhelical tension mediated by the continuous negative supercoiling action of a topoisomerase II. Cockerill and Garrard (1986) proposed a model in which topoisomerase II is involved both in binding matrix attachment sequences and in introducing and maintaining torsional stress.

It was recently demonstrated in *D. discoideum* that SDS-treatment of nuclei both from exponentially growing and from differentiating cells caused cleavages at 0.2, at 2.2 and at two sites about 17 kb upstream from the transcription unit. These cleavages have been ascribed to topoisomerase action since topoisomerases are the only agents currently known which cut DNA upon SDS addition. As additional evidence, part of the SDS-induced DNA products appear to be strongly associated with protein (Ness et al., 1986). SDS apparently induced double-stranded cleavages, indicating the presence of a topoisomerase II. However, Novobiocin, a topoisomerase II inhibitor, had no effect on the cleavage. It is therefore not clear what kind of topoisomerase(s) is/are involved (Ness et al., 1986). Comparative studies show that SDS-induced sites at -200 and at -2300 bp (Ness et al., 1986) occur at the same positions as HSS1 and HSS3. The double-stranded cleavages caused by putative topoisomerase action in *D. discoideum* rDNA (Ness et al., 1986) can so far not be functionally related to transcription or replication. The correlation of hypersensitive sites with gene activity (Elgin, 1981; 1984) suggests a possible involvement of the putative topoisomerase(s) in transcription. The association of the topoisomerase-like activities with HSS1 and HSS3 in *D. discoideum* is unlikely to be an artefact resulting from the greater accessibility of the DNA in these chromatin regions, since both differentiation stages analysed show the same SDS-induced cleavages (Ness et al., 1986) but only slug chromatin is nuclease hypersensitive at these sites.

4.2.1 SDS-INDUCED CLEAVAGES IN A REGION OF TOPOISOMERASE I CONSENSUS SEQUENCES

At least two SDS-inducible cuts were resolved at ~200 bp from the initiation site of transcription in a *D. discoideum* rDNA region which is nuclease hypersensitive in slug cells. Direct and inverted repeats of a high affinity topoisomerase I binding sequence clustered at DNase I HSS in *Tetrahymena* r-chromatin occur in this rDNA region (Gocke et al., 1983; Bonven et al. 1985). In *Tetrahymena*, the topoisomerase I cleavages are located at 0.1, 0.6 and 1.1 kb upstream from the transcription initiation site and 0.1 kb downstream from the termination site. Experiments are

underway to determine if a topoisomerase I or II is involved in the SDS-induced cleavages in *D. discoideum* (Ness, personal communication).

4.2.2 SDS-INDUCED CLEAVAGE AT THE 5' STRUCTURAL BOUNDARY

In *D. discoideum* one of the putative topoisomerase sites is located at the 5' structural boundary described above. Lawson et al. (1982) have proposed that during the differentiation process, differentiation-specific genes in various cell types are incorporated into loops which constitute DNase I sensitive domains. Hence, it is tempting to postulate that topoisomerase II sites occur in chromosomal loop anchorage regions. In *D. discoideum* topoisomerase-like activity could be detected in both differentiation stages analysed, so the structural boundary may act as constitutive matrix attachment point (Mirkowitch et al., 1984; Cockerill and Garrard, 1986). Labhart et al. (1984) proposed that the rDNA in nucleoli of *D. discoideum* is not attached to a tightly binding matrix structure, but that nucleoli are stabilized by side to side contacts between chromatin fibers. But the high salt procedure used in the course of these experiments could have disrupted salt dependent interactions (Mirkowitch et al., 1984). If the linear rDNA molecules in *D. discoideum* are anchored in vivo, a topoisomerase located at the structural boundary of the active domain would be ideally placed to actively regulate the topological state of domains and hence their transcriptional activity (Villeponteau et al., 1984; Cockerill and Garrard, 1986; North, 1985). Further topoisomerase sites of action might serve as swivel points relieving torsional strain produced by the transcriptional machinery traversing the coding region. For defining superhelical domains by matrix attachment sites, at least two attachment points are needed. The HSS mapped at the 5' (this work) and 3' end (Edwards and Firtel, 1984) of the coding region as well as the SDS-inducible cuts (Ness et al., 1986) are candidates for these points. We have not as yet analysed the 3' end of the rRNA gene, but the electron microscopic data (Sogo et al., 1984) suggest a sharp boundary, analogous to the one at the 5' end, approximately 2 kb beyond the transcribed sequence. When rRNA synthesis is low in slugs, a 1 kb stretch of bulk DNA in the vicinity to the boundary became accessible to MNase cleavage. The meaning of this structural transition is unknown. According to Cockerill and Garrard (1986) it could, for example, be argued that this feature reflects structural alterations due to a modulation of the topological state of chromatin. Since the major promoter element of rRNA transcription in yeast lies 2 kb upstream from the initiation site (Elion and Warner, 1984), the region concerned could also be directly involved in transcriptional regulation. Alternatively, the 1 kb DNA region showing the structural transition could be involved in replication (Pardoll et al., 1980; Goldberg et al., 1983; Kuenzler et al., 1984) or in sequestration of genes in the nucleolus thereby, regulating their accessibility to packing proteins.

4.3 STRUCTURAL MODEL FOR THE 5' END OF THE rRNA GENES

The actual model for the structural organization of the 5' end of *D. discoideum* rDNA is summarized in Fig. 15.

4.3.1 VEGETATIVE CELLS WITH HIGH rRNA SYNTHESIS

The coding region of rRNA genes in vegetative cells, highly active in rRNA synthesis, contains rows of transcription complexes so tightly packed that nucleosomes cannot form between them (Ness et al., 1983; Labhart et al., 1983; Sogo et al., 1984). The 5' NTS up to the structural boundary is nucleosome-free. We cannot rule out the possibility that in this as well as in the coding region the nucleosomes somehow remain attached to the otherwise unfolded DNA. It is unknown whether any transcription is initiated upstream from the normal start site of transcription as it appears to be the case for the *Xenopus* (Moss, 1983; De Winter et al., 1986; Reeder, 1984) and *Drosophila* (Miller et al., 1982; Murtif and Rae, 1985) rRNA genes. At -2.3 kb upstream from the transcription initiation site, a structural change occurs. About 1kb of rDNA in the chromatin at -2.3 to -3.3 kb shows a reduced accessibility to MNase as compared to the same region in differentiated slug cell chromatin. In addition, the nuclease cleavage pattern in this region in vegetative cells is completely different to the one of slugs, shows however site-specific phasing (Edward and Firtel, 1984) which is typical for *D. discoideum* rDNA bulk chromatin. The DNA further upstream from this region (more than 1kb beyond the -2.3 kb boundary) is covered by phased nucleosomes which are probably folded into a chromatin suprastructure.

At the rRNA initiation site, trans-acting factors interact with the rDNA. A glucocorticoid receptor binding sequence is located precisely at the start site of transcription. Topoisomerase-like activity is found at -200 and -2300 bp upstream from the start site of transcription. The rDNA region at -200 bp contains inverted and direct repeats of a topoisomerase I high affinity binding sequence (Bonven et al., 1985). The topoisomerase-like activity at -2300 bp denotes the upstream limit of the functional rDNA domain. No sequence data are available for this rDNA region.

4.3.2 DIFFERENTIATED SLUG CELLS WITH REDUCED rRNA SYNTHESIS

In differentiating slug cells, the coding region reverts to a regular nucleosomal arrangement (Ness et al., 1983; Schmidlin and Bettler, results not shown). These nucleosomes are arranged either randomly or in many different phases. The situation is more complicated for the 2.3 kb of DNA upstream from the transcribed region. In slug cells, this 2.3 kb NTS resembles the coding region in reverting to a randomly or multiply phased nucleosomal arrangement. Three strong DNase I/MNase HSS are found at -200 (HSS1), -1700 (HSS2), and -2300 bp (HSS3), respectively. The appearance of HSS has been correlated with developmental and tissue-specific gene activation. Although the structural basis for nuclease hypersensitivity is still unclear, most evidence indicates

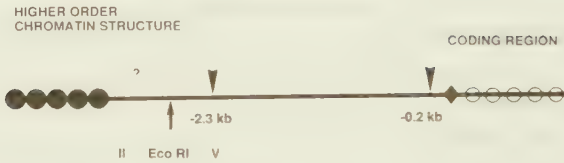
that these sites are completely free of nucleosomes (reviewed by Elgin, 1981, 1984; Igo-Kemenes et al., 1982). However, the appearance of the HSS found in slug cells seem to be a consequence of the packing of DNA into chromatin. Certainly, no HSS are found in the corresponding region of vegetative cells which is completely nucleosome-free (Schmidlin and Bettler, results not shown). Beyond the 5' boundary, the nucleosomal filament is probably folded into a tight chromatin suprastructure. Apparently, when transcription is reduced, nucleosomes reform along the active (nucleosome-sensitive) domain. Unlike the nucleosomes of the highly organized bulk chromatin these nucleosomes are not phased. It seems that non-phased nucleosomes still allow transcription to proceed, albeit at a lower rate. A phased nucleosomal organization may be transcriptionally silent.

When gene activity is reduced, there may be a decrease in the concentration (availability) of trans-acting factors. Alternatively, these factors may be modified (inactivated) or their binding influenced by other factors.

As discussed above, topoisomerase-like activity may also be involved in gene regulation. However, the two activities associated with HSS1 and HSS3 do not change when transcription is reduced.

No indirect end-labelling experiments were performed with cells of intermediate developmental stages. However, the psoralen-crosslinking experiments indicate that the whole nuclease sensitive active domain undergoes gradual changes during the progressive shut-down of rRNA synthesis..

VEGETATIVE CELLS
(HIGHEST rRNA TRANSCRIPTION RATE)



DIFFERENTIATED SLUG CELLS
(REDUCED rRNA TRANSCRIPTION RATE)

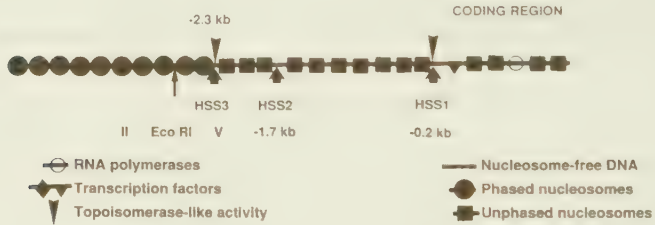


Fig. 15: Model of the structural changes in the 5' end of the rRNA genes in *D. discoideum*. Although transcription complexes may bind upstream of the initiation site, they are only shown associated with the coding region.

5. SUMMARY

In exponentially growing vegetative cells of *D. discoideum* the rRNA genes are strongly transcribed. During the differentiation cycle the rRNA synthesis decreases and in the slug stage it is low. We have used nuclease digestions and psoralen-crosslinking to compare the chromatin structure of the 36S rRNA genes in vegetative and slug cells.

Monitoring the accessibility of rDNA in chromatin to psoralen led to the identification of two distinct structural organizations upstream of the coding region. Structural changes accompanying cell differentiation could also be ascertained. The indirect-endlabelling technique was used to precisely map the boundary between the domains identified in psoralen-crosslinking experiments. This boundary is located 2.3 kb upstream from rRNA initiation site and is defined by a sharp change in the susceptibility of chromatin DNA to digestion by MNase and DNase I. Furthermore, the MNase cleavage pattern of chromatin DNA versus naked DNA changes at the boundary. We have demonstrated that the 5' boundary of the active (nuclease-sensitive) domain persists throughout cell differentiation, regardless of the transcriptional activity of the rRNA genes. MNase digestions revealed that some limited structural transitions occur in the nuclease-resistant chromatin in the vicinity of the boundary when gene activity is reduced.

In slug cells, where rRNA synthesis is greatly reduced, three MNase I/DNase I hypersensitive sites (HSS) were mapped 0.2 (HSS1), 1.7 (HSS2) and 2.3 kb (HSS3) upstream from the start site of transcription. Site-specific topoisomerase-like activity (double-stranded SDS-inducible cleavage) can be mapped to the HSS1 and HSS3 at -0.2 and -1.7 kb respectively. In the sequenced rDNA region of HSS1 inverted and direct repeats of a high affinity topoisomerase I binding sequence occur. HSS3 in r-chromatin is located at the 5' structural boundary. Hence, topoisomerase-like activity denotes the upstream limit of the active rDNA domain. We discuss the implications of this localization of topoisomerase activity on the functional organization of chromatin.

The nuclease digestion and the psoralen-crosslinking data are incorporated in a model of the rDNA chromatin structure. Changes in the 5' end of the rRNA genes related to transcriptional activity are included.

ZUSAMMENFASSUNG

Exponentiell wachsende vegetative Zellen von *D. discoideum* haben eine hohe rRNA-Syntheserate. Während des Zelldifferenzierungszyklus nimmt die rRNA-Synthese ab und ist im sogenannten Slug-Stadium, verglichen mit der maximalen Syntheserate, um 70-80% reduziert. Anhand einerseits mit Nukleasen verdauter, andererseits mit Psoralen gecrosslinkter Zellkerne haben wir die Chromatinstruktur der rRNA-Gene in vegetativen und differenzierten Zellen verglichen.

Eine Untersuchung der Psoralen-Zugänglichkeit der rDNA in isolierten Zellkernen liess auf zwei voneinander verschiedene Organisationsformen des vor dem 36S-rRNA-Gen gelegenen Chromatins schliessen. Während des Differenzierungsprozesses konnten zudem kontinuierliche strukturelle Veränderungen vor und im Gen nachgewiesen werden. Mit der indirekten Endmarkierungsmethode lokalisierten wir die Grenze zwischen den zwei erwähnten Organisationsformen des Chromatins. Sie liegt 2.3 kb vor der rRNA-Initiationsstelle und ist charakterisiert durch eine abrupte Aenderung der Zugänglichkeit des Chromatins, sowohl für *Micrococcus*-Nuklease (MNase) als auch für Deoxyribonuklease I (DNase I). Im Vergleich der beiden MNase-Verdaumuster von Chromatin-DNA und nackter DNA zeigt sich ein Unterschied, der sich von der erwähnten 5' Grenze über das inaktive (nukleaseresistente) Chromatin erstreckt. Die 5' Grenze des aktiven (nukleasesensitiven) Chromatins bleibt, unabhängig von der transkriptionellen Aktivität der rRNA-Gene, erhalten. Bei reduzierter Genaktivität finden räumlich begrenzte strukturelle Aenderungen auf der nukleasenresistenten Seite der Grenze statt.

Im Slug-Stadium, d.h. bei stark reduzierter rRNA-Synthese, treten drei hypersensitive Schnittstellen (HSS) für MNase und DNase I im Abstand von 0.2 (HSS1), 1.7 (HSS2) und 2.3 kb (HSS3) zur Initiationsstelle der rRNA-Transkription auf. In den DNA-Regionen der HSS1 und HSS3 kann eine sequenzspezifische, auf die Anwesenheit einer Topoisomerase hinweisende Aktivität festgestellt werden. Die DNA-Sequenz im Bereich der HSS1 ist bekannt. Sie enthält direkte und gegenläufige Wiederholungseinheiten (direct and inverted repeats) einer Erkennungssequenz der Topoisomerase I. Die HSS3 befindet sich an der 5' Grenze. Eine auf Topoisomerasen hindeutende Aktivität bestimmt demzufolge die vor dem Gen liegende Grenze des nukleasesensitiven Chromatins. Wir diskutieren die Bedeutung dieser räumlichen Konstellation für die funktionelle Strukturierung des Chromatins.

Die vorliegenden Ergebnisse wurden in einem Modell zusammengefasst, welches die Aenderungen der Chromatinstruktur vor rRNA Genen in Beziehung zu deren transkriptioneller Aktivität setzt.

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7. ABBREVIATIONS

bp	Base pair
Ci	Curie
DBM	Diazobenzylloxymethyl
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
EDTA	Ethylendiamine tetraacetic acid
ETS	External transcribed sequence
HSS	Hypersensitive site(s)
kb	Kilobase
ITS	Internal transcribed sequence
MNase	Micrococcal nuclease
mRNA	Messenger RNA
NaCl	Sodium chloride
NTS	Non-transcribed spacer sequence
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate
Tris	Tris(hydroxymethyl)aminomethane
V	Voltage

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